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ASSAYS FOR DETECTION OF
BACILLUS ANTHRACIS

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BACKGROUND OF THE INVENTION

Field of the Invention

This invention pertains to the field of assays for detecting *Bacillus anthracis*, the causative agent of anthrax.

Background

Anthrax spores were first produced as weapons in the 1950s. Several countries including the former Soviet Union, the United States and Iraq are known to have produced anthrax weapons. Anthrax is a particularly fearsome biological warfare agent, not only because of its deadliness, but also because anthrax weapons are relatively easy to produce and deliver. Production of anthrax spores requires little more than basic laboratory equipment and growth media. Anthrax weapons are comprised of an anthrax source and an industrial sprayer that can produce aerosol particles of the appropriate size for victims to inhale. Such sprayers, for instance, can be mounted on low flying airplanes or other vehicles and used to spread anthrax over a wide area. Because of the ease and relatively small expense involved in producing and delivering anthrax weapons, such weapons are potentially highly attractive weapons of mass destruction for terrorist groups. Thus, in addition to potential organized military conflicts that may give rise to the use of such weapons, terrorist organizations are a potential threat for the use of such weapons in airports, office buildings and other centers of human activity.

Anthrax is caused by *Bacillus anthracis*, a gram-positive, sporulating bacillus. *B. anthracis* is a soil bacterium and is distributed worldwide. The organism exists in the infected host as a vegetative bacillus and in the environment as a spore. The anthrax spore is typically the infective form of the bacterial life cycle. Anthrax spores can survive adverse environmental conditions and can remain viable for decades. Animals such as cattle,

sheep, goats and horses can contract the spores while grazing. Humans can contract anthrax from inoculation of minor skin lesions with spores from infected animals, their hides, wool or other products, such as infected meat (Franz *et al.* (1997) *J. Am. Med. Assoc.* 278(5): 399-411).

5   The typical mode of entry of the anthrax spore into the body, inhalation, results in an illness known as woolsorter's disease. After deposit in the lower respiratory tract, spores are phagocytized by tissue macrophages and transported to hilar and mediastinal lymph nodes. The spores germinate into vegetative bacilli, producing a necrotizing hemorrhagic mediastinitis (Franz *et al.*, supra). Symptoms include fever, malaise and

10   fatigue, which can easily be confused with the flu. The disease may progress to an abrupt onset of severe respiratory distress with dyspnea, stridor, diaphoresis and cyanosis. Death usually follows within 24 to 36 hours.

          Because the effects of exposure to anthrax are not immediate, and because the initial symptoms are easily confused with the flu, there is a need for a fast method to detect

15   *B. anthracis* in an environment where *B. anthracis* may have been released. This need is enhanced by the increasing number of anthrax threats that are called into governmental authorities each year. A fast method for determining whether public places have been exposed to anthrax spores in therefore essential.

          Anthrax spores have S-layers, as do spores of many other archea and bacteria.

20   Most S-layers are comprised of repeats of a single protein (Etienne-Toumelin *et al.*, *J. Bacteriol.* 177:614-20 (1995)). The S-layer of *B. anthracis*, however, is comprised of at least two proteins: EA1 (Mesnage *et al.*, *Molec. Microbiol.* 23:1147-55 (1997)) and surface array protein (SAP) (*see* Etienne-Toumelin, *et al.*, *supra*). Fully virulent *B. anthracis* isolates are encapsulated by a capsule that encompasses the S-layer of the bacteria and prevents

25   access of antibodies to both EA1 and SAP (Mesnage *et al.*, *J. Bacteriol.* 180:52-58 (1998)).

          Several methods for detecting *B. anthracis* have been reported, although none are optimal for quick and reliable detection of anthrax contamination. Detection methods include those based on amplification of nucleic acids that are specific for *B. anthracis* (Lee, *J. Appl. Microbiol.* 87:218-23 (1999); Patra, G., *FEMS Immunol. Med. Microbiol.* 15:223-31

30   (1996); Ramisse *et al*, *FEMS Microbiol. Lett.* 145(1):9-15 (1996); Bruno and Keil, *Biosens. Bioelectron.* 14:457-64 (1999); and Japanese Patent Nos. 11004693; 6261759; 6253847; and

6253846). The need to conduct time-consuming laboratory procedures to use these amplification methods limits their usefulness for quick identification of anthrax contamination. Other detection methods involve detecting spore-based epitopes of *B. anthracis* using antibodies (Yu, H., *J. Immunol. Methods* 218:1-8 (1998); Phillips *et al.*, *J.*

5   *Appl. Bacteriol.* 64:47-55 (1988); Phillips *et al.*, *FEMS Microbiol. Immunol.* 1:169-78 (1988)). Other reported detection methods include an enzyme-linked lectinosorbent assay (Graham *et al.*, *Eur. J. Clin. Microbiol.* 3:210-2 (1984)) and a method using DNA aptamers that bind anthrax spores (Bruno *et al.*, *Biosens Bioelectron.* 14(5):457-64 (1999)).

      Previous antibody-based detection methods for *B. anthracis* employed

10   antibodies raised against whole anthrax spores. Such immunogens lead to the production of antibodies that cross-react with other related bacterial species. Longchamps *et al.*, for instance, found that no antibody analyzed in their study was completely specific in recognizing anthrax spores (*J. Applied Microbiology* 87:246-49 (1999)). At least one study has shown that polyclonal antibodies raised against *B. anthracis* whole spore suspensions do

15   not react with SAP protein (Mesnage *et al*, *Molec. Microbiol.* 23:1147-55 (1997)). Closely related bacteria that may cross react with non-specific antibodies include *B. cereus*, *B. thuringiensis* and *B. mycoides* (Longchamp *et al.*, *supra.*; Phillips *et al.*, *FEMS Microbiol. Immunol.* 47:169-78 (1988)). This high degree of cross-reactivity is highly problematic for detection of anthrax because these non-toxic cross-reactive strains are widespread. *B.*

20   *thuringiensis* in particular is commonly found in the soil, in part because the bacteria is sprayed on crops for its insecticidal qualities.

      Therefore, a need exists for improved methods for detecting *Bacillus anthracis* in the environment. Such methods should be not only provide rapid results, but also should have little or no cross-reactivity with related species that are prevalent in nature.

25   The present invention fulfills this and other needs.

SUMMARY OF THE INVENTION

      The present invention provides novel methods of detecting *Bacillus anthracis*. The methods involve contacting a test sample with a capture reagent that can bind to *Bacillus anthracis* surface array protein (SAP), wherein the capture reagent forms a

30   complex with SAP if SAP is present in the test sample, and detecting whether SAP is bound

to the capture reagent. The capture reagent, for instance, can form a complex with the surface array protein if the surface array protein is present in the sample. Presence of the surface array protein is indicative of the presence of *B. anthracis* in the sample. In one embodiment, SAP comprises a polypeptide with the amino acid sequence shown in SEQ ID

5 NO:1. In another embodiment, the *B. anthracis* strain is encapsulated.

The capture reagent can comprise an antibody that binds to SAP. In some embodiments, the antibody can be a recombinant antibody, such as a recombinant polyclonal or monoclonal antibody.

In a preferred embodiment, the test sample is collected from a site of

10 suspected or threatened anthrax contamination. In another preferred embodiment, the test sample is collected using a cyclonic device. The test sample does not need to be cultured prior to contacting with the capture reagent.

In some methods of the invention, the capture reagent can be immobilized on a solid surface, such as a microtiter dish. The capture reagent can be immobilized on the

15 solid support prior to contacting the capture reagent with the test sample.

In presently preferred embodiments, the assay methods of the invention are highly sensitive. For instance, in one embodiment, antibodies of the invention used according to the methods of the invention can detect *B. anthracis* at concentrations at least as low as 10,000 cfu/ml. In a more preferred embodiment, the methods of the invention are

20 capable of detecting *B. anthracis* at concentrations at least as low as 5,000 cfu/ml. In still more preferred embodiments, the methods of the invention are capable of detecting *B. anthracis* at concentrations at least as low as 1,800 cfu/ml.

In some embodiments, SAP is detected by contacting SAP with a detection reagent that can bind SAP. Like the capture reagent, the detection reagent can be an

25 antibody that binds SAP. For instance, the detection reagent can bind a different epitope of SAP than the capture agent binds. In some embodiments, the detection reagent comprises a detectable label. The detectable label can be, for instance, a radioactive label, a fluorophore, a dye, an enzyme or a chemiluminescent label.

The invention also provides devices and kits for detecting *B. anthracis*. The

30 kits typically include, *inter alia*, a solid support upon which is immobilized a capture reagent which binds to a SAP of *B. anthracis*, and a detection reagent which binds to the SAP. In

some embodiments the solid support is a microtiter dish. In another embodiment, the capture reagent is an antibody, such as a recombinant polyclonal or monoclonal antibody or mixtures thereof. The kit can also include written instructions for using the kit to determine whether a test sample contains *B. anthracis*. In some embodiments, the kit also comprises a

5   positive control that comprises a polypeptide that comprises an antigenic determinant of *B. anthracis* SAP. The SAP can be, for example, the amino acid sequence displayed in SEQ ID NO:1.

The invention also provides for recombinant polyclonal antibody preparations that specifically bind to an antigenic determinant of *B. anthracis* SAP. For instance, the

10   SAP polypeptide can be the amino acid sequence displayed in SEQ ID NO:1.

DETAILED DESCRIPTION

Definitions

The phrase "capture reagent" refers to a molecule that specifically binds to a specific target molecule. For instance, the target molecule can be a surface array protein

15   (SAP) of *Bacillus anthracis*, or a portion thereof. Capture reagents include antibodies as well as naturally and non-naturally-occurring molecules that can specifically bind a target molecule. For instance, peptides that specifically bind a target molecule and are developed using phage display or other combinatorial system are encompassed by this definition.

A "test sample" is a sample obtained from a non-laboratory source that is not

20   known to contain *B. anthracis*. For example, a sample grown on laboratory growth media or purified from laboratory growth media is not a test sample unless it is not known whether the sample contains *B. anthracis*.

The phrases "specifically binds to" or "specifically immunoreactive with", when referring to an antibody or other binding moiety refers to a binding reaction which is

25   determinative of the presence of a target antigen in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated assay conditions, the specified binding moieties bind preferentially to a particular target antigen and do not bind in a significant amount to other components present in a test sample. Specific binding to a target antigen under such conditions may require a binding moiety that is selected for its

30   specificity for a particular target antigen. A variety of immunoassay formats may be used to

select antibodies that are specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with an antigen. *See* Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a

5   description of immunoassay formats and conditions that can be used to determine specific immunoreactivity. Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background. Specific binding between an antibody or other binding agent and an antigen generally means a binding affinity of at least 10^6 M^{-1}. Preferred binding agents bind with affinities of at least about 10^7

10   M^{-1}, and preferably 10^8 M^{-1} to 10^9 M^{-1} or 10^{10} M^{-1}.

The term "epitope" means an antigenic determinant that is capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

15   Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. Epitopes can include non-contiguous amino acids, as well as contiguous amino acids.

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one

20   "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are

25   classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. (*See generally, Fundamental Immunology* (*See, e.g.*, Paul, *Fundamental Immunology*, 3rd Ed., 1993, Raven

30   Press, New York).

The variable regions of each light/heavy chain pair form the antibody binding site. The chains all exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarily determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework

5   regions, enabling binding to a specific epitope. CDR and FR residues are delineated according to the standard sequence definition of Kabat et al., *supra*. An alternative structural definition has been proposed by Chothia *et al*. (1987) *J. Mol. Biol*. 196: 901-917; (1989) *Nature* 342: 878-883; and (1989) *J. Mol. Biol*. 186: 651-663.

The term "antibody" is used to mean whole antibodies and binding fragments

10   thereof. Binding fragments include single chain fragments, Fv fragments and Fab fragments. The term Fab fragment is sometimes used in the art to mean the binding fragment resulting from papain cleavage of an intact antibody. The terms Fab' and F(ab')$_2$ are sometimes used in the art to refer to binding fragments of intact antibodies generated by pepsin cleavage. Here, "Fab" is used to refer generically to double chain binding fragments of intact

15   antibodies having at least substantially complete light and heavy chain variable domains sufficient for antigen-specific bindings, and parts of the light and heavy chain constant regions sufficient to maintain association of the light and heavy chains. Usually, Fab fragments are formed by complexing a full-length or substantially full-length light chain with a heavy chain comprising the variable domain and at least the CH1 domain of the

20   constant region.

An "isolated" species or population of species means an object species (*e.g.*, binding polypeptides of the invention) that is the predominant species present (*i.e.*, on a molar basis it is more abundant than other species in the composition). Preferably, an isolated species comprises at least about 50, 80 or 90 percent (on a molar basis) of all

25   macromolecular species present. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods).

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that

30   are the same or have a specified percentage of nucleotides or amino acid residues that are the

same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase "substantially identical," in the context of two nucleic acids, refers to two or more sequences or subsequences that have at least 80%, preferably 85%, most preferably 90-95% nucleotide identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. For amino acid sequences, "substantially identical" refers to two or more sequences or subsequences that have at least 60% identity, preferably 75% identity, and more preferably 90-95% identify, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the nucleic acid or amino acid sequences that is at least about 10 residues in length, more preferably over a region of at least about 20 residues, and most preferably the sequences are substantially identical over at least about 100 residues. In a most preferred embodiment, the sequences are substantially identical over the entire length of the specified regions (*e.g.*, coding regions).

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally, Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between

Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1995 Supplement) (Ausubel)).

Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-410 and Altschuel *et al.* (1977) *Nucleic Acids Res.* 25: 3389-3402, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

A further indication that two nucleic acids or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross-reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where

the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described below.

"Conservatively modified variations" of a particular polynucleotide sequence

5   refers to those polynucleotides that encode identical or essentially identical amino acid sequences, or where the polynucleotide does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine.

10   Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent substitutions" or "silent variations," which are one species of "conservatively modified variations." Every polynucleotide sequence described herein which encodes a polypeptide also describes every possible silent variation, except where

15   otherwise noted. Thus, silent substitutions are an implied feature of every nucleic acid sequence which encodes an amino acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine and UGG, the only codon for tryptophan) can be modified to yield a functionally identical molecule by standard techniques. In some embodiments, the nucleotide sequences that encode the

20   enzymes are preferably optimized for expression in a particular host cell (*e.g.,* yeast, mammalian, plant, fungal, and the like) used to produce the enzymes.

Similarly, "conservative amino acid substitutions," in one or a few amino acids in an amino acid sequence are substituted with different amino acids with highly similar properties are also readily identified as being highly similar to a particular amino

25   acid sequence, or to a particular nucleic acid sequence which encodes an amino acid. Such conservatively substituted variations of any particular sequence are a feature of the present invention. Individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are "conservatively modified variations" where the

30   alterations result in the substitution of an amino acid with a chemically similar amino acid.

Conservative substitution tables providing functionally similar amino acids are well known in the art. *See, e.g.*, Creighton (1984) *Proteins*, W.H. Freeman and Company.

Description of the Preferred Embodiments

The present invention provides novel kits and methods for detecting the

5   presence or absence of *B. anthracis* in a test sample. The kits and methods are a rapid, accurate and cost-effective means for detecting *B. anthracis*. The methods involve, in presently preferred embodiments, contacting a test sample with a capture reagent that can bind to *B. anthracis* SAP. The capture reagent then forms a complex with the SAP if it is present in the sample. The SAP is then detected to determine whether the test sample

10 contains *B. anthracis*. Typically, detection is accomplished using a detection reagent that specifically binds to *B. anthracis* SAP. Both capture reagents and the detection reagents typically use binding moieties that can bind to SAP.

Unlike previously available anthrax detection methods, the methods and kits of the invention are highly sensitive. The assays and kits, in presently preferred

15 embodiments, can detect *B. anthracis* when present in a sample at a concentration of about 10^4 cfu/ml or less. Preferably, the detection limit for *B. anthracis* will be about 5×10^3 cfu/ml or less, more preferably about 1.8×10^3 cfu/ml or less, and still more preferably about 10^3 cfu/ml or less.

Moreover, the methods and kits are highly specific for *B. anthracis*. Unlike

20 previously available methods, the methods and kits of the present invention do not suffer from cross-reactivity with non-anthrax microorganisms. Previous methods of detecting *B. anthracis* relied on antibodies raised against whole anthrax spores, so these assays suffer from significant cross-reactivity. In contrast, the assays of the present invention use binding reagents that are directed to a *B. anthracis* antigen that is specific for *B. anthracis*. This

25 antigen is secreted and can be deposited on the surface of anthrax spores and other particles, for example, during the preparation of anthrax-based biological weapons. Thus, in addition to the high specificity of the detection methods of the invention, the methods are more efficient and easy to use because there is no need to disrupt the anthrax spores for binding reagents to bind their antigens. Nor must samples suspected of containing *B. anthracis* be

30 cultured prior to testing.

A. **Binding Moieties that Specifically Bind *B. anthracis* Surface Array Protein**

The assays of the invention involve detecting the presence in a test sample of a *B. anthracis* SAP polypeptide, which is an antigen that is specific for *B. anthracis*. The assays for detecting SAP are, in some embodiments, binding assays. In these assays, which include immunoassays, SAP is immobilized on a solid support using a capture reagent that can specifically bind to SAP. The immobilized SAP is then detected using detection reagents that also are capable of specifically binding to SAP. The detection reagents typically include at least a binding moiety and a detectable label.

The invention provides binding reagents that are capable of specifically binding to the SAP antigen. These binding reagents can be used in one or more steps of the assay. For example, the binding reagents can be immobilized on a solid support and used to immobilize SAP on the solid support; such immobilized binding reagents are referred to herein as "capture reagents." Binding reagents can also be used to detect *B. anthracis* antigens by, for example, attaching a detectable label to a binding moiety that binds to SAP. Suitable binding moieties include any molecule that is capable of specifically binding to SAP. Antibodies and fragments thereof are examples of binding components that are suitable for use in detection moieties.

1. Types of binding moieties

The invention provides binding moieties (or reagents) that can specifically bind *B. anthracis* SAP polypeptides. Binding reagents can also be, for example, antibodies prepared using as immunogens natural, recombinant or synthetic polypeptides derived from *B. anthracis* SAP. The amino acid sequence of a *B. anthracis* SAP is shown as SEQ ID NO:1. Such polypeptides can function as immunogens that can be used for the production of monoclonal or polyclonal antibodies. Immunogenic peptides derived from SAP can also be used as immunogens; such peptides are sometimes conjugated to a carrier polypeptide prior to inoculation. Naturally occurring, recombinantly produced, or synthetic peptides or polypeptides are suitable for use as immunogens. These can be used in either pure or impure form. Production of antibodies against SAP polypeptides of the invention is discussed in more detail below. Suitable binding moieties also include those that are obtained using methods such as phage display.

Various procedures known in the art can be used for the production of antibodies that specifically bind to SAP. For the production of polyclonal antibodies, one can use SAP to inoculate any of various host animals, including but not limited to rabbits, mice, rats, sheep, goats, and the like. The SAP polypeptide can be prepared by recombinant means

5   as described above using an expression vector containing a nucleic acid that encodes the *B. anthracis* SAP. For example, a nucleotide sequence encoding a *B. anthracis* SAP beginning at approximately 30 amino acids from the published N-terminus (i.e., at the presumed cleavage sequence) is presented in SEQ ID NO:2.

Monoclonal antibodies can be prepared by any technique that provides for the

10   production of antibody molecules by continuous cell lines in culture, including the hybridoma technique originally developed by Kohler and Milstein ((1975) *Nature* 256: 495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.* (1983) *Immunology Today* 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.* (1985) in *Monoclonal Antibodies and Cancer Therapy*,

15   Alan R. Liss, Inc., pp. 77-96). Monoclonal antibodies also can be produced in germ-free animals as was described in PCT/US89/02545 (Publication No. WO8912690, published December 12, 1989) and US Patent No. 5,091,512.

Fragments of antibodies are also useful as binding moieties. While various antibody fragments can be obtained by the digestion of an intact antibody, one of skill will

20   appreciate that such fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term "antibody," as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies (*e.g.*, single chain Fv). Single chain antibodies are also useful to construct detection moieties. Methods for producing

25   single chain antibodies were described in, for example, US Patent No. 4,946,778. Techniques for the construction of Fab expression libraries were described by Huse *et al.* (1989) *Science* 246: 1275-1281; these techniques facilitate rapid identification of monoclonal Fab fragments with the desired specificity for SAP. Suitable binding moieties also include those that are obtained using methods such as phage display.

30   To prepare a suitable antigen preparation, one can prepare an expression library from *B. anthracis* and screen the library with a polyclonal antibody that is raised

against a crude preparation of SAP. The inserts from those expression plasmids that express the SAP are then subcloned and sequenced. The SAP-encoding inserts are cloned into an expression vector and used to transform *E. coli* or other suitable host cells. The resulting preparation of recombinant SAP is then used to inoculate an animal, *e.g.*, a mouse.

5   In preferred embodiments, the binding reagents are recombinantly produced polyclonal or monoclonal antibodies that bind to SAP. Recombinant antibodies are typically produced by immunizing an animal with SAP, obtaining RNA from the spleen or other antibody-expressing tissue of the animal, making cDNA, amplifying the variable domains of the heavy and light immunoglobulin chains, cloning the amplified DNA into a phage display

10   vector, infecting *E. coli*, expressing the phage display library, and selecting those library members that express an antibody that binds to SAP. Methods suitable for carrying out each of these steps are described in, for example US patent application Ser. No. 08/835,159, filed April 4, 1997. In preferred embodiments, the antibody or other binding peptides are expressed on the cell surface of a replicable genetic unit, such as a filamentous phage, and

15   especially phage M13, Fd and F1. Most work has inserted libraries encoding polypeptides to be displayed with either pIII or pVIII of these phage, forming a fusion protein which is displayed on the surface of the phage. *See, e.g.*, Dower, WO 91/19818; Devlin, W0 91/18989; MacCafferty, WO 92/01047 (gene III); Huse, W0 92/06204; Kang, WO 92/18619 (gene VIII).

20   In a preferred embodiment, the genes that encode the heavy and light chains of antibodies present in the cDNA library are amplified using a set of primers that can amplify substantially all of the different heavy and light chains. The resulting amplified fragments that result from the amplification step are pooled and subjected to asymmetric PCR so that only one strand (*e.g.*, the antisense strand) is amplified. The single strand

25   products are phosphorylated, annealed to a single-stranded uracil template (*e.g.*, the vector BS45, described in US patent application Ser. No. 08/835,159, which has coding regions for the constant regions of mouse heavy and light chains), and introduced into a uracil DNA glycosylase[+] host cell to enrich for vectors that contain the coding sequences for heavy and light chain variable domains.

30   To screen for phage that express an antibody that binds to SAP, one can attach a label to SAP using methods known to those of skill in the art. In a preferred

embodiment, the phage that display such antibodies are selected using SAP to which is attached an immobilizable tag, *e.g.*, biotin. The phage are contacted with the biotinylated antigen, after which the phage are selected by contacting the resulting complex with avidin attached to a magnetic latex bead or other solid support. The selected phage are then plated, and may be screened with SAP to which is attached a detectable label.

In a preferred embodiment, the library is enriched for those phage that display more than one antibody that binds to SAP. Methods and vectors that are useful for this enrichment are described in US patent application Ser. No. 08/835,159. The panning can be repeated one or more times to enhance the specificity and sensitivity of the resulting antibodies. Preferably, panning is continued until the percentage of functional positives is at least about 70%, more preferably at least about 80%, and most preferably at least about 90%.

A recombinant anti-SAP monoclonal antibody can then be selected by amplifying antibody-encoding DNA from individual plaques, cloning the amplified DNA into an expression vector, and expressing the antibody in a suitable host cell (*e.g.*, *E. coli*). The antibodies are then tested for ability to bind SAP.

Recombinant polyclonal antibodies are particularly preferred because of the various forms of SAP that may be found in clinical samples due to, for example, proteolysis. The diverse fine binding specificity of members of a population of polyclonal antibodies often allows the population to bind to several forms of SAP (*e.g.*, species variants, escape mutant forms, proteolytic fragments) to which a monoclonal reagent may be unable to bind. Methods for producing recombinant polyclonal antibodies are described in US patent application Ser. No. 08/835,159, filed April 4, 1997. Specific methods of producing recombinant polyclonal antibodies that bind to SAP are described in the Examples below.

Polyclonal antibodies can be prepared as described above, except that an individual antibody is not selected. Rather, the pool of phage is used for the screening, preferably using an equal number of phage from each sample. In preferred embodiments, the phage are enriched for those that display more than one copy of the respective antibodies. The phage are then selected for those that bind to SAP. For example, one can use a biotinylated anti-SAP monoclonal antibody and SAP to concentrate those phage that express antibodies that bind to SAP. The biotinylated monoclonal antibody is immobilized on a solid support (*e.g.*, magnetic latex) to which is attached avidin. The phage that are bound to the

immobilized SAP are eluted, plated, and the panning repeated until the desired percentage of functional positives is obtained.

2. Detection reagents of the invention

The presence of SAP is generally detected using a detection reagent that is composed of a binding moiety that specifically binds to SAP. Suitable binding moieties are discussed above. The detection reagents are either directly labeled, *i.e.*, comprise or react to produce a detectable label, or are indirectly labeled, *i.e.*, bind to a molecule that is itself labeled with a detectable label. Labels can be directly attached to or incorporated into the detection reagent by chemical or recombinant methods.

In one embodiment, a label is coupled to a molecule, such as an antibody that specifically binds to SAP, through a chemical linker. Linker domains are typically polypeptide sequences, such as poly-gly sequences of between about 5 and 200 amino acids. In some embodiments, proline residues are incorporated into the linker to prevent the formation of significant secondary structural elements by the linker. Preferred linkers are often flexible amino acid subsequences that are synthesized as part of a recombinant fusion protein comprising the RNA recognition domain. In one embodiment, the flexible linker is an amino acid subsequence that includes a proline, such as Gly(x)-Pro-Gly(x) where x is a number between about 3 and about 100. In other embodiments, a chemical linker is used to connect synthetically or recombinantly produced recognition and labeling domain subsequences. Such flexible linkers are known to persons of skill in the art. For example, poly(ethylene glycol) linkers are available from Shearwater Polymers, Inc. Huntsville, Alabama. These linkers optionally have amide linkages, sulfhydryl linkages, or heterofunctional linkages.

The detectable labels used in the assays of the present invention, which are attached to the detection reagent, can be primary labels (where the label comprises an element that is detected directly or that produces a directly detectable element) or secondary labels (where the detected label binds to a primary label, *e.g.*, as is common in immunological labeling). An introduction to labels, labeling procedures and detection of labels is found in Polak and Van Noorden (1997) *Introduction to Immunocytochemistry*, 2nd ed., Springer Verlag, NY and in Haugland (1996) *Handbook of Fluorescent Probes and*

Research Chemicals, a combined handbook and catalogue Published by Molecular Probes,
Inc., Eugene, OR. Patents that described the use of such labels include U.S. Patent Nos.
3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

5   Primary and secondary labels can include undetected elements as well as
detected elements. Useful primary and secondary labels in the present invention can include
spectral labels such as green fluorescent protein, fluorescent dyes (*e.g.*, fluorescein and
derivatives such as fluorescein isothiocyanate (FITC) and Oregon Green™, rhodamine and
derivatives (*e.g.*, Texas red, tetrarhodimine isothiocynate (TRITC), etc.), digoxigenin, biotin,
phycoerythrin, AMCA, CyDyes™, and the like), radiolabels (e.g., ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P,
10 etc.), enzymes (e.g., horse radish peroxidase, alkaline phosphatase etc.), spectral colorimetric
labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene,
latex, etc.) beads. The label can be coupled directly or indirectly to a component of the
detection assay (*e.g.*, the detection reagent) according to methods well known in the art. As
indicated above, a wide variety of labels may be used, with the choice of label depending on
15 sensitivity required, ease of conjugation with the compound, stability requirements, available
instrumentation, and disposal provisions.

         Preferred labels include those that use: 1) chemiluminescence (using
horseradish peroxidase and/or alkaline phosphatase with substrates that produce photons as
breakdown products as described above) with kits being available, *e.g.*, from Molecular
20 Probes, Amersham, Boehringer-Mannheim, and Life Technologies/ Gibco BRL; 2) color
production (using both horseradish peroxidase and/or alkaline phosphatase with substrates
that produce a colored product (kits available from Life Technologies/Gibco BRL, and
Boehringer-Mannheim)); 3) fluorescence using, *e.g.*, an enzyme such as alkaline
phosphatase, together with the substrate AttoPhos (Amersham) or other substrates that
25 produce fluorescent products, 4) fluorescence (*e.g.*, using Cy-5 (Amersham), fluorescein,
and other fluorescent tags); 5) radioactivity. Other methods for labeling and detection will
be readily apparent to one skilled in the art.

         For use of the present invention outside the laboratory, preferred labels are
non-radioactive and readily detected without the necessity of sophisticated instrumentation.
30 Preferably, detection of the labels will yield a visible signal that is immediately discernable
upon visual inspection. One preferred example of detectable secondary labeling strategies

uses an antibody that recognizes SAP in which the antibody is linked to an enzyme (typically by recombinant or covalent chemical bonding). The antibody is detected when the enzyme reacts with its substrate, producing a detectable product. Preferred enzymes that can be conjugated to detection reagents of the invention include, *e.g.*, β-galactosidase, luciferase,

5 horse radish peroxidase, and alkaline phosphatase. The chemiluminescent substrate for luciferase is luciferin. One embodiment of a fluorescent substrate for β-galactosidase is 4-methylumbelliferyl-β-D-galactoside. Embodiments of alkaline phosphatase substrates include p-nitrophenyl phosphate (pNPP), which is detected with a spectrophotometer; 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) and fast red/napthol

10 AS-TR phosphate, which are detected visually; and 4-methoxy-4-(3-phosphonophenyl) spiro[1,2-dioxetane-3,2'-adamantane], which is detected with a luminometer. Embodiments of horse radish peroxidase substrates include 2,2'azino-bis(3-ethylbenzthiazoline-6 sulfonic acid) (ABTS), 5-aminosalicylic acid (5AS), o-dianisidine, and o-phenylenediamine (OPD), which are detected with a spectrophotometer; and 3,3,5,5'-tetramethylbenzidine (TMB),

15 3,3'diaminobenzidine (DAB), 3-amino-9-ethylcarbazole (AEC), and 4-chloro-1-naphthol (4C1N), which are detected visually. Other suitable substrates are known to those skilled in the art. The enzyme-substrate reaction and product detection are performed according to standard procedures known to those skilled in the art and kits for performing enzyme immunoassays are available as described above.

20 The presence of a label can be detected by inspection, or a detector which monitors a particular probe or probe combination can be used to detect the detection reagent label. Typical detectors include spectrophotometers, phototubes and photodiodes, microscopes, scintillation counters, cameras, film and the like, as well as combinations thereof. Examples of suitable detectors are widely available from a variety of commercial

25 sources known to persons of skill. Commonly, an optical image of a substrate comprising bound labeling moieties is digitized for subsequent computer analysis.

B. **_B. anthracis_ Protein Surface Array Protein (SAP) Nucleic Acids and Polypeptides**

The binding reagents used in the assays and kits of the invention are generally

30 obtained using a *B. anthracis* SAP polypeptide as an immunogen. The entire SAP can be

used, or polypeptide subfragments that include an immunogenic epitope can be used. Suitable SAP immunogens can be isolated from *B. anthracis* cultures, or more preferably can be produced using recombinant methods.

1.   SAP Polypeptides

5   SAP polypeptides can be produced by methods known to those of skill in the art. The amino acid sequence of a *B. anthracis* SAP polypeptide is provided as SEQ ID NO:1. A *B. anthracis* SAP polypeptide from a different strain is described in Etienne-Toumelin *et al.*, *J. Bacteriol.* 177:614-620 (1995).

In a presently preferred embodiment, the SAP proteins, or immunogenic

10   subsequences thereof, are synthesized using recombinant DNA methodology. Generally this involves creating a DNA sequence that encodes the polypeptide, modified as desired, placing the DNA in an expression cassette under the control of a particular promoter, expressing the protein in a host, isolating the expressed protein and, if required, renaturing the protein.

15   SAP polypeptides can be expressed in a variety of host cells, including *E. coli*, other bacterial hosts, yeasts, filamentous fungi, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines. Techniques for gene expression in microorganisms are described in, for example, Smith, *Gene Expression in Recombinant Microorganisms* (*Bioprocess Technology*, Vol. 22), Marcel Dekker, 1994.

20   Examples of bacteria that are useful for expression include, but are not limited to, *Escherichia, Enterobacter, Azotobacter, Erwinia, Bacillus, Pseudomonas, Klebsiella, Proteus, Salmonella, Serratia, Shigella, Rhizobia, Vitreoscilla*, and *Paracoccus*. Filamentous fungi that are useful as expression hosts include, for example, the following genera: *Aspergillus, Trichoderma, Neurospora, Penicillium, Cephalosporium, Achlya,*

25   *Podospora, Mucor, Cochliobolus*, and *Pyricularia*. *See, e.g.*, US Patent No. 5,679,543 and Stahl and Tudzynski, Eds., *Molecular Biology in Filamentous Fungi*, John Wiley & Sons, 1992. Synthesis of heterologous proteins in yeast is well known and described in the literature. *Methods in Yeast Genetics*, Sherman, F., *et al.*, Cold Spring Harbor Laboratory, (1982) is a well recognized work describing the various methods available to produce the

30   enzymes in yeast.

SAP proteins, whether recombinantly or naturally produced, can be purified, either partially or substantially to homogeneity, according to standard procedures of the art, such as, for example, ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (*see*, generally, R. Scopes, *Protein*
5 *Purification*, Springer-Verlag, N.Y. (1982), Deutscher, *Methods in Enzymology Vol. 182: Guide to Protein Purification.*, Academic Press, Inc. N.Y. (1990)). Once purified, partially or to homogeneity as desired, the polypeptides can then be used (*e.g.,* as immunogens for antibody production).

One of skill in the art would recognize that after chemical synthesis,
10 biological expression, or purification, the SAP protein(s) may possess a conformation substantially different than the native conformations of the constituent polypeptides. In this case, it may be necessary or desirable to denature and reduce the polypeptide and then to cause the polypeptide to re-fold into the preferred conformation. Methods of reducing and denaturing proteins and inducing re-folding are well known to those of skill in the art (*See,*
15 Debinski *et al.* (1993) *J. Biol. Chem.* 268: 14065-14070; Kreitman and Pastan (1993) *Bioconjug. Chem.* 4: 581-585; and Buchner *et al.* (1992) *Anal. Biochem.* 205: 263-270). Debinski *et al.*, for example, describe the denaturation and reduction of inclusion body proteins in guanidine-DTE. The protein is then refolded in a redox buffer containing oxidized glutathione and L-arginine.

20 One of skill also would recognize that modifications can be made to the SAP polypeptides without diminishing their immunogenic activity. Some modifications can be made to facilitate the cloning, expression, or incorporation of the polypeptide into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional
25 amino acids (*e.g.,* poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

2. *B. anthracis* SAP-encoding nucleic acids

Nucleic acids that encode *B. anthracis* are useful for the recombinant production of SAP. Such nucleic acids can be isolated, for example, by routine cloning
30 methods. The cDNA sequence provided in SEQ ID NO:2 can be used to provide probes that

specifically hybridize to a SAP gene, to a SAP mRNA, or to a SAP cDNA in a cDNA library (*e.g.,* in a Southern or Northern blot). Once the target SAP nucleic acid is identified, it can be isolated according to standard methods known to those of skill in the art (*see, e.g.,* Sambrook, Berger, and Ausubel, *supra.*).

5   SAP nucleic acids also can be isolated by amplification methods such as polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (SSR). A wide variety of cloning and *in vitro* amplification methodologies are well-known to persons of skill. Examples of these techniques and instructions sufficient to direct persons of skill

10   through many cloning exercises are found in Berger, Sambrook, and Ausubel (all *supra.*); Cashion *et al.*, U.S. patent number 5,017,478; and Carr, European Patent No. 0,246,864. Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.* (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.*,

15   eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3: 81-94; (Kwoh *et al.* (1989) *Proc. Nat'l. Acad. Sci. USA* 86: 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87: 1874; Lomell *et al.* (1989) *J. Clin. Chem.* 35: 1826; Landgren *et al.* (1988) *Science* 241: 1077-1080; Van Brunt (1990) *Biotechnology* 8: 291-294; Wu and Wallace (1989) *Gene,* 4:

20   560; and Barringer *et al.* (1990) *Gene* 89: 117.

A polynucleotide that encodes a SAP polypeptide can be operably linked to appropriate expression control sequences for a particular host cell in which the polypeptide is to be expressed. Such constructs are often referred to as "expression cassettes." For *E. coli*, appropriate control sequences include a promoter such as the T7, trp, or lambda

25   promoters, a ribosome binding site and preferably a transcription termination signal. For eukaryotic cells, the control sequences typically include a promoter which optionally includes an enhancer derived from immunoglobulin genes, SV40, cytomegalovirus, *etc.*, and a polyadenylation sequence, and may include splice donor and acceptor sequences. In yeast, convenient promoters include GAL1,10 (Johnson and Davies (1984) *Mol. Cell. Biol.* 4:1440-

30   1448) ADH2 (Russell *et al.* (1983) *J. Biol. Chem.* 258:2674-2682), PHO5 (*EMBO J.* (1982) 6:675-680), and MFα1 (Herskowitz and Oshima (1982) in *The Molecular Biology of the*

Yeast Saccharomyces (eds. Strathern, Jones, and Broach) Cold Spring Harbor Lab., Cold Spring Harbor, N.Y., pp. 181-209).

Expression cassettes are typically introduced into a vector that facilitates entry into a host cell, and maintenance of the expression cassette in the host cell. Vectors that include a polynucleotide that encodes a SAP polypeptide are provided by the invention. Such vectors often include an expression cassette that can drive expression of the SAP polypeptide. To easily obtain a vector of the invention, one can clone a polynucleotide that encodes the SAP polypeptide into a commercially or commonly available vector. A variety of common vectors suitable for this purpose are well known in the art. For cloning in bacteria, common vectors include pBR322 derived vectors such as pBLUESCRIPT™, and λ-phage derived vectors. In yeast, vectors include Yeast Integrating plasmids (*e.g.*, YIp5) and Yeast Replicating plasmids (the YRp series plasmids) and pGPD-2. A multicopy plasmid with selective markers such as Leu-2, URA-3, Trp-1, and His-3 is also commonly used. A number of yeast expression plasmids such as YEp6, YEp13, YEp4 can be used as expression vectors. The above-mentioned plasmids have been fully described in the literature (Botstein *et al.* (1979) *Gene* 8:17-24; Broach *et al.* (1979) *Gene*, 8:121-133). For a discussion of yeast expression plasmids, *see, e.g.*, Parents, B., *YEAST* (1985), and Ausubel, Sambrook, and Berger, *all supra*). Expression in mammalian cells can be achieved using a variety of commonly available plasmids, including pSV2, pBC12BI, and p91023, as well as lytic virus vectors (*e.g.,* vaccinia virus, adenovirus, and baculovirus), episomal virus vectors (*e.g.,* bovine papillomavirus), and retroviral vectors (*e.g.,* murine retroviruses).

The nucleic acids that encode SAP polypeptides can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for *E. coli* and calcium phosphate treatment or electroporation for *E. coli* or mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the *amp, gpt, neo* and *hyg* genes, among others. Techniques for transforming fungi are well known in the literature and have been described, for instance, by Beggs *et al.* ((1978) *Proc. Natl. Acad. Sci. USA* 75: 1929-1933), Yelton *et al.* ((1984) *Proc. Natl. Acad. Sci. USA* 81: 1740-1747), and Russell ((1983) *Nature* 301: 167-169). Procedures for transforming yeast are also well known (*see, e.g.,* Beggs (1978) *Nature* (London), 275:104-109; and Hinnen *et al.* (1978) *Proc. Natl. Acad. Sci. USA*,

75:1929-1933. Transformation and infection methods for mammalian and other cells are described in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor

5   Laboratory, Cold Spring Harbor Press, NY, (Sambrook *et al.*); *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.,* eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel).

C.   Assay Formats

10   The *B. anthracis* detection methods of the present invention can be carried out in a wide variety of assay formats. Typically, the assay methods involve immobilization of *B. anthracis* SAP on a solid support, followed by detection of the immobilized SAP. The detectable labels can be detected directly after immobilization on the solid support, for example, or indirectly by an enzymatic or other reaction that results in a detectable change in

15   a reactant that is present in the detection assay reaction.

1. ELISA detection methods of the invention

               Presently preferred assay systems for use in the kit and methods of the invention are based on the enzyme-linked immunosorbent assay (ELISA) method. General methods for ELISA are well known to those of skill in the art (*see, e.g.,* Elder *et al.*, *J. Clin.*

20   *Microbiol.* 16:141 (1982); Ausubel *et al.*, *supra*). Generally, antigens fixed to a solid surface are detected using antigen-specific antibodies that are detected by way of an enzymatic reaction. In a presently preferred embodiment, the ELISA method used is the "sandwich" method wherein the antigens are bound to the solid surface via an antibody bound to the solid surface. A second antibody, typically linked to an enzyme, is then contacted to the

25   antigen, washed, then contacted with the enzyme substrate to select binding. These and other embodiments of the ELISA method are taught in, for example, Ausubel *et al.* § 11.2, *supra*.

               To immobilize SAP on the solid support, a capture reagent that specifically binds to SAP is non-diffusively associated with the support. The capture reagents can be non-diffusively immobilized on the support either by covalent or non-covalent methods,

30   which are known to those of skill in the art. *See, e.g.*, Pluskal *et al.* (1986) *BioTechniques* 4:

272-283. Suitable supports include, for example, glasses, plastics, polymers, metals, metalloids, ceramics, organics, and the like. Specific examples include, but are not limited to, microtiter plates, nitrocellulose membranes, nylon membranes, and derivatized nylon membranes, beads, and also particles, such as agarose, SEPHADEX™, and the like. Assay

5   systems for use in the methods and kits of the invention include, but are not limited to, dipstick-type devices, immunochromatographic test strips and radial partition immunoassay devices, microtiter assays and flow-through devices. Conveniently, where the solid support is a membrane, the test sample can flow through the membrane, for example, by gravity, capillary action, or under positive or negative pressure.

10   Once the test sample has been contacted with the solid support, the solid support is then contacted with detection reagents for SAP. The solid support can be washed prior to contact with detection reagents to remove unbound reagents and test sample components. After incubation of the detection reagents for a sufficient time to bind a substantial portion of the immobilized SAP, any unbound labeled reagents are removed by,

15   for example, washing. The detectable label associated with the detection reagents is then detected. For example, in the case of an enzyme used as a detectable label, a substrate for the enzyme that turns a visible color upon action of the enzyme is placed in contact with the bound detection reagent. A visible color will then be observed in proportion to the amount of the specific antigen in the sample.

20   *2. Membrane-based detection methods of the invention*

In some embodiments, the assay methods are carried out using a membrane-based detection system such as are described in U.S. Patent No. 5,922,615 and EP 447154. These systems employ an apparatus that includes a porous member, such as a membrane or a filter, onto which is bound a multiplicity of capture reagents that specifically bind *B.*

25   *anthracis* SAP. The apparatus also includes a non-absorbent member with a textured surface in communication with the lower surface of the porous member. The textured surface of the non-absorbent member can be a grooved surface (*e.g.*, analogous to the surface of a record album) or it can be composed of channels, such that when the porous and non-absorbent members are brought into contact with one another a network of capillary channels is

30   formed. The capillary network is formed from the contact of the porous member with the

textured surface of the non-absorbent member and can be constructed either before or subsequent to the initial contacting of the porous member with a fluid.

In some embodiments, the capillary communication between the porous member and the non-absorbent member favors delaying the transferal of fluid from the porous member to the capillary network formed by the porous member and the textured surface of the non-absorbent member until the volume of the added fluid substantially exceeds the void volume of the porous member. The transferal of fluid from the porous member to the network of capillary channels formed by the porous member and the textured surface of the non-absorbent member can occur without the use of external means, such as positive external pressure or vacuum, or contact with an absorbent material.

The devices of the present invention can also include an optional member which is placed in contact with the upper surface of the porous member and may be used to partition the upper surface of the device into discrete openings. Such openings can access either the porous member or the textured surface of the non-absorbent second member. The optional member can in conjunction with the non-absorbent member compose a fluid receiving zone in which there is no intervening porous member. A fluid receiving zone constructed from the non-absorbent member and the optional member provides fluid capacity in addition to that provided by the network of capillary channels created by the contact of the porous member and the non-absorbent member. The openings in the optional member may include a first fluid opening and also an additional fluid opening. The first fluid opening functions as a portal for the introduction of the first fluid added to the device. The additional fluid opening serves as an additional portal through which additional fluids may be added to the inventive device.

To perform an assay using these devices, a volume of the test sample is added to the porous member, where the sample permeates the void volume of the porous member and thereby contacts the anchor moieties immobilized on the porous member. In a non-competitive assay, the sample to be assayed is applied to the porous member and the SAP, if present, is bound by the anchor moieties. A detection reagent for SAP is then added as an additional fluid; these bind to the complex of SAP and capture reagent. Alternatively, the detection reagent can be added to the sample prior to application of the sample to the porous member so that the binding of detection reagent to SAP occurs prior to the binding of SAP to

the capture reagent. In another embodiment, the capture reagent and detection reagent are added to the sample, after which the complex of capture reagent, SAP, and detection reagent binds to a binding agent that is either combined with these reagents or is immobilized on the porous member. An additional fluid containing reagents to effect a separation of free from

5 bound labeled reagents can be added to remove excess detection reagent, if needed.

This device is designed to provide sufficient sensitivity to measure low concentrations of SAP because one can use large amounts of sample and efficiently remove the excess of detection reagent. Indeed, the efficient separation of free from bound label achieved by the network of capillary channels of this device improves the discrimination of

10 specific SAP-associated signal over non-specific background signal. If needed, a signal developer solution is then added to enable the label of the detection moiety to develop a detectable signal. The signal developed can then be related to the concentration of the target ligand within the sample. In a preferred embodiment, the transfer of fluid between the porous first member of the device and the network of capillary channels formed by the

15 contact of the porous member and textured surface of the non-absorbent second member of the device is generally self-initiated at the point when the total volume of fluid added to the device exceeds the void volume of the porous member, thus obviating the need for active interaction by the user to remove excess fluid from the analyte detection zone. The point at which the fluid transfer is initiated is dependent upon the objectives of the assay. Normally,

20 it is desirable to contact the sample with all of the zones on the porous member which contain immobilized receptor. This method enables the detection of SAP in a manner that is simple, rapid, convenient, sensitive and efficient in the use of reagents.

Competitive binding assays can also be used to detect SAP. Conveniently, these assays are performed using the described devices by adding to a sample a labeled

25 analog of SAP. The labeled analog and SAP present in the sample compete for the binding sites of the capture reagents. Alternatively, the capture reagents can be combined with the sample and labeled analogs with subsequent immobilization of the capture reagents onto the porous member through contact with a binding agent. An additional fluid to separate the free from bound label may be added to the device, followed if needed by a signal development

30 solution to enable detection of the label of the labeled analog which has complexed with

capture reagent immobilized on the porous member. The amount of labeled SAP bound to the porous member is related to the concentration of SAP in the sample.

D.   Kits for Detecting Anthrax

This invention also provides kits for the detection and/or quantification of anthrax using the methods described herein. The kits can include a container containing one or more of the above-discussed detection reagents with or without labels, and capture reagents, either free or bound to solid supports. A suitable solid support, such as a membrane, can also be included in the kits of the invention. The kits can provide the solid supports in the form of an assay apparatus that is adapted to use in the described assay. Preferably, the kits will also include reagents used in the described assays, including reagents useful for detecting the presence of the detectable labels. Other materials useful in the performance of the assays can also be included in the kits, including test tubes, transfer pipettes, and the like. The kits can also include written instructions for the use of one or more of these reagents in any of the assays described herein.

The kits of the invention can also include an internal and/or an external control. An internal control can consist of the SAP polypeptide. The control antigen can conveniently be preattached to a capture reagent in a zone of the solid support adjacent to the zone to which the sample is applied. The external control can also consist of the SAP polypeptide. Typically, the antigen present in the external control will be at a concentration at or above the sensitivity limit of the assay means. The external control antigen can be diluted in the sample diluent and assayed in the same manner as would a biological sample. Alternatively, the external control SAP polypeptide can be added to an aliquot of an actual biological sample to determine the sensitivity of the assay. The kits of the present invention can contain materials sufficient for one assay, or can contain sufficient materials for multiple assays.

E.   Test Samples

Samples to test for the presence of anthrax can be collected from any potential source of anthrax. Samples can be collected from, for example, the air, water, food, soil or other solids or liquids. In one embodiment, the methods and kits of the invention can

be used to determine if terrorists have planted anthrax in a public area. In preferred embodiments, it is unknown whether the test sample contains *B. anthracis*.

Air samples can be collected using, for example, a cyclonic collection device (*see, e.g.*, Jensen *et al.*, *Am. Ind. Hyg. Assoc. J.* 53:660-67 (1992); Cage *et al.*, *Ann. Allergy Asthma Immunol.* 77:401-6 (1996)). Such a device can capture a volume of air, submit the air to turbulence such that any particles in the air (*e.g.*, anthrax spores or SAP-coated particles) are deposited on a moist surface. Typically, air flowing through cyclonic tubes forms a vortex in the tube that induces high centrifugal forces on particles in the air (Anderson *et al.*, *Johns Hopkins APL Technical Digest* 20(3) (1999)). The rotational forces segregate the larger particle to the outside of the tube. Variations in the tube diameter, length, taper angle and flow velocity determine particle separation size. Particles can then be captured by letting the particles slide down the tube walls into a filter bag or by washing the walls with a liquid and capturing the concentrate. The objects can then be collected and analyzed for the presence of anthrax. A variety of cyclonic devices are discussed in, e.g., Maddox *et al. Monthly Microscopical J.* 286-290 (1870); Fisher-Klosterman, Inc. Product Bulletin 218-C, 2900 West Broadway, Louisville, KY.; Hering, "Impactors, Cyclones, and Other Inertial and Gravitational Collectors," in *Air Sampling Instruments for Evaluation of Atmospheric contaminants*, 8th Ed., American Conference of Governmental Industrial Hygienists, Cincinnati, OH, 279-321 (1995) and; Stoutas, *et al. J. Aerosol Sci.* 25(7):1321-1330 (1994). Handheld air samplers can also be used to obtain samples that are tested according to the methods of the invention (*see, e.g.*, Kenny *et al.*, *Am. Ind. Hyg. Assoc. J.* 59:831-41 (1998)). Sampling of solid or liquid objects is known to those skilled in the art.

Several cyclonic collection devices are known, including conventional impactors and virtual impactors. Conventional impactors work by directing the particle-containing air through a nozzle onto a collection plate. A variation of the conventional impactor is the virtual impactor, which operates by directing the air stream from the nozzle to an opening with a restricted flow. Larger particles enter an opening which forms a virtual surface, and become entrained in a minor flow or reduced velocity, while smaller particles follow the major flow. The virtual impactor has the benefit of concentrating particle quantity from low density in the high volume flow to high density in the low volume flow. *See, e.g.*, Anderson *et al.*, *supra*.

A significant advantage of the assay methods and kits of the invention is that the sensitivity is such that a sample need not be cultured prior to assay. This not only provides a faster and less expensive assay, but also makes it possible to obtain a result in the field. Samples need not be sent to a laboratory facility for processing. This is particularly advantageous in military situations, in which suitable laboratory facilities may not be available.

EXAMPLES

The following examples are offered to illustrate, but not to limit the present invention.

Example 1

Isolation of a gene encoding *Bacillus anthracis* surface array protein (SAP)

This Example describes the cloning and characterization of a gene that encodes a *Bacillus anthracis* surface array protein (SAP).

Isolation of B. anthracis DNA

Bacillus anthracis genomic DNA isolated from the non-pathologic Sterne strain was used as a template source for PCR amplification of a nucleic acid that codes for SAP. A total of 6 ml of *Bacillus anthracis* Sterne strain (1×10^{10}/ml in PBS pH 7.4) was pelleted in a microcentrifuge at 10,000g for 5 minutes. Bacterial pellets were then combined and resuspended in a final volume of 1 ml lysis buffer (50 mM Tris(hydroxymethyl) aminomethane ("Tris") pH 7.8, 10 mM ethylenediaminetetraacetic acid ("EDTA"), 100 μg/ml Ribonuclease (RNase A) (Roche Molecular Biochemical, Indianapolis, Ind.), 0.5% Triton X-100™ (T-octylphenoxypolyethoxyethanol) (Sigma, St. Louis, MO), 12.5% sucrose). Lysozyme (Sigma) was added to a final concentration of 2 mg/ml and the mixture incubated for 1 hr at 37°C. 300 μg of Proteinase K (Roche Molecular Biochemical, Indianapolis, Ind.) and a one-tenth volume of 10% SDS was added to the mixture followed by a 1 hr incubation at 56°C. NaCl was then added to a final concentration of 500 mM by adding one-tenth volume of 5 M NaCl. The mixture was then twice extracted with phenol/chloroform (phenol:chloroform:isoamyl alcohol (50:49:1)) and the DNA in the aqueous layer sheared by passing the solution through an 18 gauge needle. DNA was

precipitated with 2.5 volumes of ethanol and resuspended in 200 µl of distilled water. This DNA preparation was extracted once with Tris pH 8 equilibrated phenol, two times with phenol/chloroform and finally twice with chloroform (chloroform:isoamyl alcohol (49:1)) alone. DNA in the aqueous layer was precipitated a final time and resuspended in 500 µl of

5   distilled water, yielding approximately 79 µg of DNA at 158 µg/ml. This DNA was used as a template in the subsequent PCR amplification of the SAP gene.

Cloning of *Bacillus anthracis* SAP gene via PCR

Appropriate PCR primers were made corresponding to the coding sequence of the 5' and 3' ends of the *B. anthracis* SAP gene (see primer sequence below). These

10   primers were based on a published nucleotide sequence (Etienne-Toumelin *et al.*, *supra*). DNA encoding the native signal sequence of SAP (amino acids 1-29) was purposefully omitted from the cloning since a functional signal sequence was provided by the expression vector pBRncoH3 (described in copending, commonly-owned US patent application Ser. No. 08/835,159, filed April 4, 1997). The 5' primer contains 23 bases of vector sequence at

15   its 5'-end that corresponds to the 3'-end of the pBRncoH3 vector. The 3' primer contains 19 bases of the tetracycline promoter, removed by *Hin*dIII digestion in the vector, in addition to 20 bases of vector sequence 3' to the *Hin*dIII site. The 3' primer was also engineered to encode a hexahistidine amino acid tag at the C-terminus of the SAP protein to allow for efficient purification using nickel-chelate affinity chromatography (see below).

20

5' PCR primer: 5' - TCGCTGCCCAACCAGCCATGGCCGCAGGTAAAA
                      CATTCCCAGAC -3' (SEQ ID NO:2)

25
3' PCR primer: 5'- GTGATAAACTACCGCATTAAAGCTTATCGATGATA
                    AGCTGTCAATTAGTGATGGTGATGGTGATGTTTTG
                    TTGCAGGTTTTGCTTCTTT -3' (SEQ ID NO:3)

The nucleic acid that encodes SAP was amplified using these primers and approximately 30 ng of *Bacillus anthracis* genomic DNA as template. The amplification was performed using Expand™ DNA polymerase (Roche Molecular Biochemical (Indianapolis,

30   Ind.). SAP insert DNA (~300 ng) was purified and annealed to the *Hin*dIII-digested pBRncoH3 vector (100 ng) at a 6:1 molar ratio of insert to vector. An aliquot was electroporated into 40µl of electrocompetent *E. coli* strain DH10B as described in Example

3. Various dilutions of the transformed cells were plated on LB agar plates supplemented with tetracycline (10μg/ml) and grown overnight at 37°C. Three colonies were each picked into 3 ml 2xYT, supplemented with tetracycline (10μg/ml), and grown overnight at 37°C. The following day, glycerol freezer stocks were made for long term storage at -80°C.

5   In order to confirm that the SAP gene had indeed been cloned, each of the three clones was tested for the ability to synthesize SAP protein upon arabinose induction as described below. All three clones produced a protein of the predicted size, approximately 94 kDa in molecular mass, and were shown to react with a rabbit anti-anthracis polyclonal serum using Western blot analysis (data not shown). Two of the three clones were

10   sequenced and compared against the National Center for Biotechnology Information's (NCBI) non-redundant nucleotide database using the BLAST search engine. This search indicated that a SAP gene had indeed been cloned. There were eight differences in the predicted amino acid sequence compared to the noted published sequence. These changes are lysine 264 to arginine, glutamic acid 478 to alanine, arginine 482 to histidine, glutamic

15   acid 496 to aspartic acid, lysine 556 to arginine, glutamic acid 606 to aspartic acid, lysine 607 to threonine, and valine 751 to alanine. Amino acid numbering is based on the published sequence (Etienne-Toumelin *et al.*, *supra*). These differences may be due to the fact that a different *Bacillus anthracis* strain was used in the work described here. The original published work did not use the Sterne strain. The predicted amino acid sequence of

20   the SAP gene cloned here shows 8 amino acid differences out of 785, and is thus 99.0% identical to the published sequence.

Example 2

Expression and purification of recombinant *Bacillus anthracis* SAP from *E. coli*

This Example describes the expression and purification of *B. anthracis* SAP

25   using *E. coli*.

A shake flask containing 2xYT supplemented with 1% glycerol was inoculated with an *E. coli* DH10B strain from Example 1 that contained a cloned *B. anthracis* SAP gene and incubated overnight in an Innova 4330 incubator shaker (New Brunswick Scientific, Edison, NJ) set at 37°C, 300 rpm. The inoculum was used to seed 500

30   mL cultures of defined medium (Pack *et al.* (1993) *Bio/Technology* 11: 1271-1277)

supplemented with 3 g/L L-leucine, 3 g/L L-isoleucine, 12 g/L casein digest (Difco, Detroit, MI), 12.5 g/L glycerol and 10 µg/ml tetracycline. Cultures were grown in 2 L Tunair shake flasks (Shelton Scientific, Shelton, CT) at 37°C and 300 rpm. Cells were grown to an optical density of approximately 4 absorption units at 600 nm. Expression of SAP was then induced by addition of L(+)-arabinose to 2 g/L during this logarithmic growth phase. The flasks were then maintained at 23°C and 300 rpm overnight.

The following morning, bacterial cultures were passed through an M-110Y Microfluidizer (Microfluidics, Newton, MA) at 17,000 psi. The homogenate was clarified in a J2-21 centrifuge (Beckman, Fullerton, CA) and recombinant SAP purified from the supernatant using immobilized metal affinity chromatography. Briefly, Chelating Sepharose FastFlow™ resin (Pharmacia, Piscataway, NJ) was charged with 0.1 M $NiCl_2$ and equilibrated in 20 mM borate, 150 mM NaCl, 10 mM imidazole, 0.01 % NaN_3, pH 8. A stock solution was used to bring the supernatant concentration to 10 mM imidazole, pH 8. Chelating resin was then added to the supernatant and the mixture shaken for 1 hour at room temperature, 150-200 rpm. During this time, SAP was captured by means of the high affinity interaction between nickel and the hexahistidine tag engineered onto the C-terminus of SAP. After 1 hour, the resin mixture was poured into a chromatography column and washed with 20 mM borate, 150 mM NaCl, 10 mM imidazole, 0.01 % NaN_3, pH 8.0. SAP was eluted from the resin with the same buffer containing 200 mM imidazole instead of 10 mM.

The volume of eluted SAP was reduced using a centrifuge concentrator with a 30 kDa molecular weight cut off (Amicon, Beverly, MA), and the sample subsequently dialyzed against sterile phosphate-buffered solution (PBS) for immunizations and BBS (20 mM borate, 150 mM NaCl, 0.01% NaN_3, pH 8.0) for biotinylation. Isolated SAP was evaluated for purity by SDS-PAGE analysis and shown to be greater than 95% pure. The protein concentration of recombinant SAP was determined by UV absorbance at 280 nm, assuming an absorbance of 0.593 for a 1 mg/ml solution.

Example 3

Construction of a phage-display library

This Example describes the construction of a phage display library from which binding reagents that are specific for *B. anthracis* SAP were identified.

5   **Immunization and mRNA Isolation**

A phage display library for identification of SAP-binding molecules was constructed as follows. A/J mice (Jackson Laboratories, Bar Harbor, ME) were immunized intraperitoneally with recombinant SAP antigen, using 100 μg protein in Freund's complete adjuvant, on day 0, and with 100 μg antigen on day 28. Test bleeds of mice were obtained

10   through puncture of the retro-orbital sinus. If, by testing the titers, they were deemed high by ELISA using biotinylated SAP antigen immobilized via neutravidin (Reacti-Bind™ NeutrAvidin™-Coated Polystyrene Plates, Pierce, Rockford, IL), the mice were boosted with 100 μg of protein on day 70, 71 and 72, with subsequent sacrifice and splenectomy on day 77. If titers of antibody were not deemed satisfactory, mice were boosted with 100 μg

15   antigen on day 56 and a test bleed taken on day 63. If satisfactory titers were obtained, the animals were boosted with 100 μg of antigen on day 98, 99, and 100 and the spleens harvested on day 105.

The spleens were harvested in a laminar flow hood and transferred to a petri dish, trimming off and discarding fat and connective tissue. The spleens were macerated

20   quickly with the plunger from a sterile 5 cc syringe in the presence of 1.0 ml of solution D (25.0 g guanidine thiocyanate (Boehringer Mannheim, Indianapolis, IN), 29.3 ml sterile water, 1.76 ml 0.75 M sodium citrate pH 7.0, 2.64 ml 10% sarkosyl (Fisher Scientific, Pittsburgh, PA), 0.36 ml 2-mercaptoethanol (Fisher Scientific, Pittsburgh, PA)). This spleen suspension was pulled through an 18 gauge needle until all cells were lysed and the viscous

25   solution was transferred to a microcentrifuge tube. The petri dish was washed with 100 μl of solution D to recover any remaining spleen. This suspension was then pulled through a 22 gauge needle an additional 5-10 times.

The sample was divided evenly between two microcentrifuge tubes and the following added, in order, with mixing by inversion after each addition: 50 μl 2 M sodium

30   acetate pH 4.0, 0.5 ml water-saturated phenol (Fisher Scientific, Pittsburgh, PA), 100μl

chloroform/isoamyl alcohol 49:1 (Fisher Scientific, Pittsburgh, PA). The solution was vortexed for 10 seconds and incubated on ice for 15 min. Following centrifugation at 14 krpm for 20 min at 2-8°C, the aqueous phase was transferred to a fresh tube. An equal volume of water saturated phenol:chloroform:isoamyl alcohol (50:49:1) was added, and the

5   tube vortexed for ten seconds. After a 15 min incubation on ice, the sample was centrifuged for 20 min at 2-8°C, and the aqueous phase transferred to a fresh tube and precipitated with an equal volume of isopropanol at -20°C for a minimum of 30 min. Following centrifugation at 14 krpm for 20 min at 4°C, the supernatant was aspirated away, the tubes briefly spun and all traces of liquid removed from the RNA pellet.

10   The RNA pellets were each dissolved in 300 μl of solution D, combined, and precipitated with an equal volume of isopropanol at -20°C for a minimum of 30 min. The sample was centrifuged 14 krpm for 20 min at 4°C, the supernatant aspirated as before, and the sample rinsed with 100 μl of ice-cold 70% ethanol. The sample was again centrifuged 14 krpm for 20 min at 4°C, the 70% ethanol solution aspirated, and the RNA pellet dried *in*

15   *vacuo*. The pellet was resuspended in 100μl of sterile diethyl pyrocarbonate-treated water. The concentration was determined by A_{260} using an absorbance of 1.0 for a concentration of 40 μg/ml. The RNAs were stored at -80°C.

Preparation of complementary DNA (cDNA)

The total RNA purified from mouse spleens as described above was used

20   directly as template for cDNA preparation. RNA (50 μg) was diluted to 100 μL with sterile water, and 10 μL of 130 ng/μL oligo dT_{12} (synthesized on Applied Biosystems Model 392 DNA synthesizer) was added. The sample was heated for 10 min at 70°C, then cooled on ice. Forty μL 5X first strand buffer was added (Gibco/BRL, Gaithersburg, MD), along with 20 μL 0.1 M dithiothreitol (Gibco/BRL, Gaithersburg, MD), 10 μL 20 mM deoxynucleoside

25   triphosphates (dNTP's, Boehringer Mannheim, Indianapolis, IN), and 10 μL water on ice. The sample was then incubated at 37°C for 2 min. Ten μL reverse transcriptase (Superscript™ II, Gibco/BRL, Gaithersburg, MD) was added and incubation was continued at 37°C for 1 hr. The cDNA products were used directly for polymerase chain reaction (PCR).

Amplification of antibody genes by PCR

To amplify substantially all of the H and L chain genes using PCR, primers were chosen that corresponded to substantially all published sequences. Because the nucleotide sequences of the amino termini of H and L contain considerable diversity, 33 oligonucleotides were synthesized to serve as 5' primers for the H chains, and 29 oligonucleotides were synthesized to serve as 5' primers for the kappa L chains as described in US Patent Application Ser. No. 08/835,159, filed April 4, 1997. The constant region nucleotide sequences for each chain required only one 3' primer for the H chains and one 3' primer for the kappa L chains.

A 50 µL reaction was performed for each primer pair with 50 pmol of 5' primer, 50 pmol of 3' primer, 0.25 µL Taq DNA Polymerase (5 units/µL, Boehringer Mannheim, Indianapolis, IN), 3 µL cDNA (prepared as described in Example 3), 5 µL 2 mM dNTP's, 5 µL 10 x Taq DNA polymerase buffer with MgCl₂ (Boehringer Mannheim, Indianapolis, IN), and H$_2$O to 50 µL. Amplification was done using a GeneAmp® 9600 thermal cycler (Perkin Elmer, Foster City, CA) with the following thermocycle program: 94°C for 1 min; 30 cycles of 94°C for 20 sec, 55°C for 30 sec, and 72°C for 30 sec; 72°C for 6 min; 4°C.

The dsDNA products of the PCR process were then subjected to asymmetric PCR using only a 3' primer to generate substantially only the anti-sense strand of the target genes. A 100 µL reaction was done for each dsDNA product with 200 pmol of 3' primer, 2 µL of ds-DNA product, 0.5 µL Taq DNA Polymerase, 10 µL 2 mM dNTP's, 10 µL 10 X Taq DNA polymerase buffer with MgCl₂ (Boehringer Mannheim, Indianapolis, IN), and H$_2$O to 100µL. The same PCR program as that described above was used to amplify the single-stranded (ss)-DNA.

Purification of single-stranded DNA by high performance liquid chromatography and kinasing single-stranded DNA

The H chain ss-PCR products and the L chain single-stranded PCR products were ethanol precipitated by adding 2.5 volumes ethanol and 0.2 volumes 7.5 M ammonium acetate and incubating at -20°C for at least 30 min. The DNA was pelleted by centrifuging in an Eppendorf centrifuge at 14 krpm for 10 min at 2-8°C. The supernatant was carefully aspirated, and the tubes were briefly spun a 2nd time. The last drop of supernatant was

removed with a pipette. The DNA was dried *in vacuo* for 10 min on medium heat. The H

chain products were pooled in 210 µL water and the L chain products were pooled separately

in 210 µL water. The single-stranded DNA was purified by high performance liquid

chromatography (HPLC) using a Hewlett Packard 1090 HPLC and a Gen-Pak™ FAX anion

5   exchange column (Millipore Corp., Milford, MA). The gradient used to purify the single-

stranded DNA is shown in Table 1, and the oven temperature was 60°C. Absorbance was

monitored at 260 nm. The single-stranded DNA eluted from the HPLC was collected in 0.5

min fractions. Fractions containing single-stranded DNA were ethanol precipitated, pelleted

and dried as described above. The dried DNA pellets were pooled in 200 µL sterile water.

10

Table 1: HPLC gradient for purification of ss-DNA

Time (min)	%A	%B	%C	Flow (ml/min)
0	70	30	0	0.75
2	40	60	0	0.75
17	15	85	0	0.75
18	0	100	0	0.75
23	0	100	0	0.75
24	0	0	100	0.75
28	0	0	100	0.75
29	0	100	0	0.75
34	0	100	0	0.75
35	70	30	0	0.75

Buffer A is 25 mM Tris, 1 mM EDTA, pH 8.0

Buffer B is 25 mM Tris, 1 mM EDTA, 1 M NaCl, pH 8.0

15   Buffer C is 40 mm phosphoric acid

        The single-stranded DNA was 5'-phosphorylated in preparation for

mutagenesis. Twenty-four µL 10X kinase buffer (United States Biochemical, Cleveland,

OH), 10.4 µL 10 mM adenosine-5'-triphosphate (Boehringer Mannheim, Indianapolis, IN),

and 2 µL polynucleotide kinase (30 units/µL, United States Biochemical, Cleveland, OH)

20   was added to each sample, and the tubes were incubated at 37°C for 1 hr. The reactions were

stopped by incubating the tubes at 70°C for 10 min. The DNA was purified with one

extraction of Tris equilibrated phenol (pH > 8.0, United States Biochemical, Cleveland,

OH):chloroform:isoamyl alcohol (50:49:1) and one extraction with chloroform:isoamyl

alcohol (49:1). After the extractions, the DNA was ethanol precipitated and pelleted as

25   described above. The DNA pellets were dried, then dissolved in 50 µL sterile water. The

concentration was determined by measuring the absorbance of an aliquot of the DNA at 260 nm using 33 µg/ml for an absorbance of 1.0. Samples were stored at -20°C.

Preparation of uracil templates used in generation of spleen antibody phage libraries

    One ml of *E. coli* CJ236 (BioRAD, Hercules, CA) overnight culture was
5   added to 50 ml 2xYT in a 250 ml baffled shake flask. The culture was grown at 37°C to $OD_{600} = 0.6$, inoculated with 10 µl of a 1/100 dilution of BS45 vector phage stock (described in US Patent Application Ser. No. 08/835,159, filed April 4, 1997) and growth continued for 6 hr. Approximately 40 ml of the culture was centrifuged at 12 krpm for 15 minutes at 4°C. The supernatant (30 ml) was transferred to a fresh centrifuge tube and incubated at room
10   temperature for 15 minutes after the addition of 15 µl of 10 mg/ml RNaseA (Boehringer Mannheim, Indianapolis, IN). The phage were precipitated by the addition of 7.5 ml of 20% polyethylene glycol 8000 (Fisher Scientific, Pittsburgh, PA)/3.5M ammonium acetate (Sigma Chemical Co., St. Louis, MO) and incubation on ice for 30 min. The sample was centrifuged at 12 krpm for 15 min at 2-8°C. The supernatant was carefully discarded, and the
15   tube briefly spun to remove all traces of supernatant. The pellet was resuspended in 400 µl of high salt buffer (300 mM NaCl, 100 mM Tris pH 8.0, 1 mM EDTA), and transferred to a 1.5 ml tube.

    The phage stock was extracted repeatedly with an equal volume of equilibrated phenol:chloroform:isoamyl alcohol (50:49:1) until no trace of a white interface
20   was visible, and then extracted with an equal volume of chloroform:isoamyl alcohol (49:1). The DNA was precipitated with 2.5 volumes of ethanol and 1/5 volume 7.5 M ammonium acetate and incubated 30 min at -20°C. The DNA was centrifuged at 14 krpm for 10 min at 4°C, the pellet washed once with cold 70% ethanol, and dried *in vacuo*. The uracil template DNA was dissolved in 30 µl sterile water and the concentration determined by A_{260} using an
25   absorbance of 1.0 for a concentration of 40 µg/ml. The template was diluted to 250 ng/µL with sterile water, aliquoted, and stored at -20°C.

Mutagenesis of uracil template with ss-DNA and electroporation into E. coli to generate antibody phage libraries

Antibody phage display libraries were generated by simultaneously introducing single-stranded heavy and light chain genes onto a phage display vector uracil template. A typical mutagenesis was performed on a 2 µg scale by mixing the following in a 0.2 ml PCR reaction tube: 8 µl of (250 ng/µL) uracil template, 8 µL of 10 x annealing buffer (200 mM Tris pH 7.0, 20 mM MgCl₂, 500 mM NaCl), 3.33 µl of kinased single-stranded heavy chain insert (100 ng/µL), 3.1 µl of kinased single-stranded light chain insert (100 ng/µL), and sterile water to 80 µl. DNA was annealed in a GeneAmp® 9600 thermal cycler using the following thermal profile: 20 sec at 94°C, 85°C for 60 sec, 85°C to 55°C ramp over 30 min, hold at 55°C for 15 min. The DNA was transferred to ice after the program finished. The extension/ligation was carried out by adding 8 µl of 10 x synthesis buffer (5 mM each dNTP, 10 mM ATP, 100 mM Tris pH 7.4, 50 mM MgCl₂, 20 mM DTT), 8 µL T4 DNA ligase (1U/µL, Boehringer Mannheim, Indianapolis, IN), 8 µL diluted T7 DNA polymerase (1U/µL, New England BioLabs, Beverly, MA) and incubating at 37°C for 30 min. The reaction was stopped with 300 µL of mutagenesis stop buffer (10 mM Tris pH 8.0, 10 mM EDTA). The mutagenesis DNA was extracted once with equilibrated phenol (pH>8):chloroform:isoamyl alcohol (50:49:1), once with chloroform:isoamyl alcohol (49:1), and the DNA was ethanol precipitated at -20°C for at least 30 min. The DNA was pelleted and the supernatant carefully removed as described above. The sample was briefly spun again and all traces of ethanol removed with a pipetman. The pellet was dried *in vacuo.* The DNA was resuspended in 4 µL of sterile water.

One microliter of mutagenesis DNA (500 ng) was transferred into 40 µl electrocompetent *E. coli* DH12S (Gibco/BRL, Gaithersburg, MD) using electroporation. The transformed cells were mixed with approximately 1.0 ml of overnight XL-1 cells which were diluted with 2xYT broth to 60% the original volume. This mixture was then transferred to a 15-ml sterile culture tube and 9 ml of top agar added for plating on a 150-mm LB agar plate. Plates were incubated for 4 hrs at 37°C and then transferred to 20°C overnight. First round antibody phage were made by eluting phage off these plates in 10 ml of 2xYT, spinning out debris, and taking the supernatant. These samples are the antibody phage display libraries used for selecting antibodies against SAP. Efficiency of the

electroporations was measured by plating 10 μl of a 10^{-4} dilution of suspended cells on LB agar plates, follow by overnight incubation of plates at 37°C. The efficiency was calculated by multiplying the number of plaques on the 10^{-4} dilution plate by 10^6. Library electroporation efficiencies are typically greater than 1×10^7 phage under these conditions.

5   **Transformation of E. coli by electroporation**

Electrocompetent *E. coli* cells were thawed on ice. DNA was mixed with 40 μL of these cells by gently pipetting the cells up and down 2-3 times, being careful not to introduce an air bubble. The cells were transferred to a Gene Pulser cuvette (0.2 cm gap, BioRAD, Hercules, CA) that had been cooled on ice, again being careful not to introduce an

10   air bubble in the transfer. The cuvette was placed in the *E. coli* Pulser (BioRAD, Hercules, CA) and electroporated with the voltage set at 1.88 kV according to the manufacturer's recommendation. The transformed sample was immediately resuspended in 1 ml of 2xYT broth or 1 ml of a mixture of 400μl 2xYT/600μl overnight XL-1 cells and processed as procedures dictated.

15   **Plating M13 phage or cells transformed with antibody phage-display vector mutagenesis reaction**

Phage samples were added to 200 μL of an overnight culture of *E. coli* XL1-Blue when plating on 100 mm LB agar plates or to 600 μL of overnight cells when plating on 150 mm plates in sterile 15 ml culture tubes. After adding LB top agar (3 ml for 100 mm

20   plates or 9 ml for 150 mm plates, top agar stored at 55°C (*see*, Appendix A1, Sambrook *et al.*, *supra.*), the mixture was evenly distributed on an LB agar plate that had been pre-warmed (37°C-55°C) to remove any excess moisture on the agar surface. The plates were cooled at room temperature until the top agar solidified. The plates were inverted and incubated at 37°C as indicated.

25   **Preparation of biotinylated SAP and biotinylated antibodies**

Concentrated recombinant SAP antigen (Example 2 above) was extensively dialyzed into BBS (20 mM borate, 150 mM NaCl, 0.1% NaN$_3$, pH 8.0). After dialysis, 1 mg of SAP (1 mg/ml in BBS) was reacted with a 15 fold molar excess of biotin-XX-NHS ester (Molecular Probes, Eugene, OR, stock solution at 40 mM in DMSO). The reaction was

incubated at room temperature for 90 min and then quenched with taurine (Sigma Chemical Co., St. Louis, MO) at a final concentration of 20 mM. The biotinylated reaction mixture was then dialyzed against BBS at 2-8°C. After dialysis, biotinylated SAP was diluted in panning buffer (40 mM Tris, 150 mM NaCl, 20 mg/ml BSA, 0.1% Tween 20, pH 7.5),

5   aliquoted, and stored at -80°C until needed.

Antibodies were reacted with 3-(N-maleimidylpropionyl)biocytin (Molecular Probes, Eugene, OR)using a free cysteine located at the carboxy terminus of the heavy chain. Antibodies were reduced by adding DTT to a final concentration of 1 mM for 30 min at room temperature. Reduced antibody was passed through a Sephadex G50 desalting column

10   equilibrated in 50 mM potassium phosphate, 10 mM boric acid, 150 mM NaCl, pH 7.0. 3-(N-maleimidylpropionyl)-biocytin was added to a final concentration of 1 mM and the reaction allowed to proceed at room temperature for 60 min. Samples were then dialyzed extensively against BBS and stored at 2-8°C.

Preparation of avidin magnetic latex

15   The magnetic latex (Estapor, 10% solids, Bangs Laboratories, Fishers, IN) was thoroughly resuspended and 2 ml aliquoted into a 15 ml conical tube. The magnetic latex was suspended in 12 ml distilled water and separated from the solution for 10 min using a magnet (PerSeptive Biosystems, Framingham, MA). While maintaining the separation of the magnetic latex with the magnet, the liquid was carefully removed using a

20   10 ml sterile pipette. This washing process was repeated an additional three times. After the final wash, the latex was resuspended in 2 ml of distilled water. In a separate 50 ml conical tube, 10 mg of avidin-HS (NeutrAvidin, Pierce, Rockford, IL) was dissolved in 18 ml of 40 mM Tris, 0.15 M sodium chloride, pH 7.5 (TBS). While vortexing, the 2 ml of washed magnetic latex was added to the diluted avidin-HS and the mixture mixed an additional 30

25   seconds. This mixture was incubated at 45°C for 2 hr, shaking every 30 minutes. The avidin magnetic latex was separated from the solution using a magnet and washed three times with 20 ml BBS as described above. After the final wash, the latex was resuspended in 10 ml BBS and stored at 4°C.

Immediately prior to use, the avidin magnetic latex was equilibrated in

30   panning buffer (40 mM Tris, 150 mM NaCl, 20 mg/ml BSA, 0.1% Tween 20, pH 7.5). The

avidin magnetic latex needed for a panning experiment (200μl/sample) was added to a sterile 15 ml centrifuge tube and brought to 10 ml with panning buffer. The tube was placed on the magnet for 10 min to separate the latex. The solution was carefully removed with a 10 ml sterile pipette as described above. The magnetic latex was resuspended in 10 ml of panning
5   buffer to begin the second wash. The magnetic latex was washed a total of 3 times with panning buffer. After the final wash, the latex was resuspended in panning buffer to the starting volume.

Example 4

Selection of Recombinant Polyclonal antibodies to *Bacillus anthracis* SAP antigen

10   Binding reagents that specifically bind to *B. anthracis* SAP were selected from the phage display libraries created from hyperimmunized mice as described in Example 3.

Panning

        First round antibody phage were prepared as described in Example 3 using
15   BS45 uracil template. Electroporations of mutagenesis DNA were performed yielding phage samples derived from different immunized mice. To create more diversity in the recombinant polyclonal library, each phage sample was panned separately.
        Before the first round of functional panning with biotinylated SAP antigen, antibody phage libraries were selected for phage displaying both heavy and light chains on
20   their surface by panning with 7F11-magnetic latex (as described in Examples 21 and 22 of US patent application Ser. No. 08/835,159, filed April 4, 1997). Functional panning of these enriched libraries was performed in principle as described in Example 16 of US patent application Ser. No. 08/835,159. Specifically, 10 μL of 1×10^{-6} M biotinylated SAP antigen was added to the phage samples (approximately 1×10^{-8} M SAP final concentration), and the
25   mixture allowed to come to equilibrium overnight at 2-8°C.
        After reaching equilibrium, samples were panned with avidin magnetic latex to capture antibody phage bound to SAP. Equilibrated avidin magnetic latex (Example 3), 200μL latex per sample, was incubated with the phage for 10 min at room temperature. After 10 min, approximately 9 ml of panning buffer was added to each phage sample, and

the magnetic latex separated from the solution using a magnet. After a ten minute separation, unbound phage was carefully removed using a 10 ml sterile pipette. The magnetic latex was then resuspended in 10 ml of panning buffer to begin the second wash. The latex was washed a total of three times as described above. For each wash, the tubes were in contact with the magnet for 10 min to separate unbound phage from the magnetic latex. After the third wash, the magnetic latex was resuspended in 1 ml of panning buffer and transferred to a 1.5mL tube. The entire volume of magnetic latex for each sample was then collected and resuspended in 200 ul 2xYT and plated on 150 mm LB plates as described in Example 3 to amplify bound phage. Plates were incubated at 37°C for 4 hr, then overnight at 20°C.

The 150 mm plates used to amplify bound phage were used to generate the next round of antibody phage. After the overnight incubation, second round antibody phage were eluted from the 150 mm plates by pipetting 10 mL of 2xYT media onto the lawn and gently shaking the plate at room temperature for 20 min. The phage samples were then transferred to 15 ml disposable sterile centrifuge tubes with a plug seal cap, and the debris from the LB plate pelleted by centrifuging the tubes for 15 min at 3500 rpm. The supernatant containing the second round antibody phage was then transferred to a new tube.

A second round of functional panning was set up by diluting 100 μL of each phage stock into 900 μL of panning buffer in 15 ml disposable sterile centrifuge tubes. Biotinylated SAP antigen was then added to each sample as described for the first round of panning, and the phage samples incubated for 1 hr at room temperature. The phage samples were then panned with avidin magnetic latex as described above. The progress of panning was monitored at this point by plating aliquots of each latex sample on 100 mm LB agar plates to determine the percentage of kappa positives. The majority of latex from each panning (99%) was plated on 150 mm LB agar plates to amplify the phage bound to the latex. The 100 mm LB agar plates were incubated at 37°C for 6-7 hr, after which the plates were transferred to room temperature and nitrocellulose filters (pore size 0.45mm, BA85 Protran, Schleicher and Schuell, Keene, NH) were overlaid onto the plaques.

Plates with nitrocellulose filters were incubated overnight at room temperature and then developed with a goat anti-mouse kappa alkaline phosphatase conjugate to determine the percentage of kappa positives as described below. Phage samples with lower percentages (< 70%) of kappa positives in the population were subjected to a

round of panning with 7F11-magnetic latex before performing a third functional round of panning overnight at 2-8°C using biotinylated SAP antigen at approximately 2×10^{-9} M. This round of panning was also monitored for kappa positives. Individual phage samples that had kappa positive percentages greater than 80% were pooled and subjected to a final round of

5   panning overnight at 2-8°C at 5×10^{-9} M SAP. Antibody genes contained within the eluted phage from this fourth round of functional panning were subcloned into the expression vector, pBRncoH3.

The subcloning process was done generally as described in Example 18 of US patent application Ser. No. 08/835,159. After subcloning, the expression vector was

10   electroporated into DH10B cells and the mixture grown overnight in 2xYT containing 1% glycerol and 10 μg/ml tetracycline. After a second round of growth and selection in tetracycline, aliquots of cells were frozen at –80°C as the source for SAP polyclonal antibody production. Two polyclonal antibodies, designated IIT004.1 and IIT005.1, were selected from two libraries derived from different sets of spleens. Monoclonal antibodies

15   were selected from these polyclonal mixtures by plating a sample of the mixture on LB agar plates containing 10 μg/ml tetracycline and screening for antibodies that recognized SAP.

Detection of alkaline phosphatase conjugates

After overnight incubation of nitrocellulose filters on LB agar plates, filters were carefully removed from the plates with membrane forceps and incubated for 2 hr in 10

20   mM TRIS, 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1% polyvinyl alcohol, 1% bovine serum albumin, 0.1% sodium azide, pH 8.0 (Block buffer). After 2 hr, the filters were incubated with goat anti-mouse kappa-AP (Southern Biotechnology Associates, Inc, Birmingham, AL) for 2-4 hours. The goat anti-mouse kappa-AP was diluted into Block buffer at a final concentration of 1 μg/ml. Filters were washed three times with 40 mM Tris,

25   150 mM NaCl, 0.05% Tween 20, pH 7.5 (TBST) for 5 min each. After the final wash, the filters were developed in a solution containing 0.2 M 2-amino-2-methyl-1-propanol (JBL Scientific, San Luis Obispo, CA), 0.5 M Tris, 0.33 mg/ml nitro blue tetrazolium ((NBT) Fisher Scientific, Pittsburgh, PA) and 0.166 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, p-toluidine salt.

Expression and purification of recombinant antibodies against SAP

        A shake flask inoculum was generated overnight from a -70°C cell bank in an Innova 4330 incubator shaker (New Brunswick Scientific, Edison, NJ) set at 37°C, 300 rpm. The inoculum was used to seed a 20 L fermentor (Applikon, Foster City, CA) containing

5   defined culture medium (Pack *et al.* (1993) *Bio/Technology* 11: 1271-1277) supplemented with 3 g/L L-leucine, 3 g/L L-isoleucine, 12 g/L casein digest (Difco, Detroit, MI), 12.5 g/L glycerol and 10 µg/ml tetracycline. The temperature, pH and dissolved oxygen in the fermentor were controlled at 26°C, 6.0-6.8 and 25 % saturation, respectively. Foam was controlled by addition of polypropylene glycol (Dow, Midland, MI). Glycerol was added to

10   the fermentor in a fed-batch mode. Fab expression was induced by addition of L(+)-arabinose (Sigma, St. Louis, MO) to 2 g/L during the late logarithmic growth phase. Cell density was measured by optical density at 600 nm in an UV-1201 spectrophotometer (Shimadzu, Columbia, MD). Following run termination and adjustment of pH to 6.0, the culture was passed twice through an M-210B-EH Microfluidizer (Microfluidics, Newton,

15   MA) at 17,000 psi. The high pressure homogenization of the cells released the Fab into the culture supernatant.

        The first step in purification was expanded bed immobilized metal affinity chromatography (EB-IMAC). Streamline™ chelating resin (Pharmacia, Piscataway, NJ) was charged with 0.1 M NiCl₂ and was then expanded and equilibrated in 50 mM acetate,

20   200 mM NaCl, 10 mM imidazole, 0.01% NaN₃, pH 6.0 buffer flowing in the upward direction. A stock solution was used to bring the culture homogenate to 10 mM imidazole, following which it was diluted two-fold or higher in equilibration buffer to reduce the wet solids content to less than 5% by weight. It was then loaded onto the Streamline column flowing in the upward direction at a superficial velocity of 300 cm/hr. The cell debris passed

25   through unhindered, but the Fab was captured by means of the high affinity interaction between nickel and the hexahistidine tag on the Fab heavy chain. After washing, the expanded bed was converted to a packed bed and the Fab was eluted with 20 mM borate, 150 mM NaCl, 200 mM imidazole, 0.01 % NaN₃, pH 8.0 buffer flowing in the downward direction.

30   The second step in the purification used ion-exchange chromatography (IEC). Q Sepharose FastFlow resin (Pharmacia, Piscataway, NJ) was equilibrated in 20 mM borate,

37.5 mM NaCl, 0.01 % NaN₃, pH 8.0. The Fab elution pool from the EB-IMAC step was diluted four-fold in 20 mM borate, 0.01 % NaN₃, pH 8.0 and loaded onto the IEC column. After washing, the Fab was eluted with a 37.5 - 200 mM NaCl salt gradient. The elution fractions were evaluated for purity using an Xcell II™ SDS-PAGE system (Novex, San

5   Diego, CA) prior to pooling. Finally, the Fab pool was concentrated and diafiltered into 20 mM borate, 150 mM NaCl, 0.01 % NaN₃, pH 8.0 buffer for storage. This was achieved in a Sartocon Slice™ system fitted with a 10,000 MWCO cassette (Sartorius, Bohemia, NY). The final purification yields were typically 50%. The concentration of the purified Fab was measured by UV absorbance at 280 nm, assuming an absorbance of 1.6 for a 1 mg/ml

10   solution.

Culture of *Bacillus* spp. and preparation of cleared culture supernatant antigen

Nonencapsulated *Bacillus anthracis*, Sterne strain was obtained from the Colorado Serum Company. *B. cereus* OH599 was the kind gift of Dr. A. Kotiranta, *B. globigii* was obtained from Dr. L. Larson, and *B. thuringiensis* 10792 was obtained from the

15   American Type Culture Collection (Manassas, VA). Organisms were cultured on tryptic soy agar containing 5% sheep blood (Hardy Diagnostics, Santa Maria, CA) or in brain heart infusion broth (Becton Dickinson and Company, Cockeysville, MD) at 37°C.

For preparation of cleared culture supernatant antigens, *B. anthracis*, Sterne strain was grown in brain heart infusion broth at 37°C with aeration for 24 h. A sample of

20   the culture was serially diluted 10-fold in sterile 0.01M phosphate buffered saline, pH 7.4 (PBS) and 100 µl of each dilution was plated on a blood agar plate for determination of the number of viable organisms. The culture was subjected to centrifugation at 10,000 x g for 20 min at 4°C using a J2-21 centrifuge (Beckman, Fullerton, CA). The supernatant was transferred to a sterile bottle and a protease inhibitor cocktail (Sigma-Aldrich, Inc) was

25   added. The sample was filtered using a 0.2 µm pore-size membrane filter unit (Millipore Corp., Bedford, MA) then dialyzed in 4 L of 0.01M PBS, pH 7.4, 2 mM EDTA, 0.1 mM phenymethylsulfonyl fluoride at 4°C with four buffer changes in 24 h. The dialyzed sample was aliquoted and stored at –80°C. The concentration of SAP in the cleared culture supernatant was quantified by a sandwich enzyme-linked immunosorbant assay using

30   purified recombinant SAP as a standard. Analysis of the amount of SAP recovered from the

culture supernatant indicated that 1 ng of SAP corresponded to approximately 2.9×10^3 organisms (i.e. 0.35 pg/organism).

Example 5

Selection of monoclonal antibodies to SAP from the recombinant polyclonal antibody mixtures

Monoclonal antibodies against SAP were isolated from clones containing the recombinant polyclonal mixtures (Example 4) by plating a diluted sample of the mixture on LB agar plates containing 10 µg/ml tetracycline. Individual colonies were then tested for the ability to produce antibody that recognized recombinant SAP using surface plasmon resonance (BIACORE) (BIACORE, Uppsala, Sweden). Small scale production of these monoclonal antibodies was accomplished using a Ni-chelate batch-binding method (see below). Antibodies isolated from this method were diluted 1:3 in HBS-EP (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v)), captured with a goat anti-mouse kappa antibody (Southern Biotechnology Associates, Inc, Birmingham, AL) coupled to a BIACORE CM5 sensor chip, and tested for the ability to bind recombinant SAP. Antibodies that bound SAP were then evaluated using BIACORE epitope mapping analysis. Antibodies that bound distinct epitopes were produced on a larger scale and then conjugated to biotin and alkaline phosphatase. These conjugates were then tested in an ELISA assay to determine the sensitivity and utility of these antibodies.

Minipreparation of monoclonal antibodies by Ni-chelate batch-binding method

Individual colonies were isolated from the recombinant polyclonal mixtures (Example 4) and used to inoculate 3 ml cultures of 2xYT medium containing 1% glycerol supplemented with 10 µg/ml tetracycline. These cultures were grown in an Innova 4330 incubator shaker (New Brunswick Scientific, Edison, NJ) set at 37°C, 300 rpm. The next morning 0.5 ml of each culture was used to inoculate shake flasks containing 50 ml of defined medium, (Pack *et al.* (1993) *Bio/Technology* 11: 1271-1277) supplemented with 3 g/L L-leucine, 3 g/L L-isoleucine, 12 g/L casein digest (Difco, Detroit, MI), 12.5 g/L glycerol and 10 µg/ml tetracycline. These cultures were shaken at 300 rpm, 37 °C until an optical density of 4 was reached at 600 nm. Fab expression was then induced by adding L(+)-arabinose (Sigma, St. Louis, MO) to 2 g/L and shifting the temperature to 23 °C with

overnight shaking. The next day the following was added to the 50 ml cultures: 0.55 ml of 1 M imidazole, 5 ml B-PER (Pierce, Rockford, IL) and 2 ml Ni-chelating resin (Chelating Sepharose FastFlow™ resin Pharmacia, Piscataway, NJ). The mixture was shaken at 300 rpm, 23 °C for 1 hour after which time shaking was stopped and the resin allowed to settle to

5   the bottom of the flasks for 15 minutes.

The supernatant was then poured off and the resin resuspended in 40 ml of BBS (20 mM borate, 150 mM NaCl, 0.1% NaN$_3$, pH 8.0) containing 10 mM imidazole. This suspension was transferred to a 50 ml conical tube and the resin washed a total of 3 times with BBS containing 10mM imidazole. Washing was accomplished by low speed

10 centrifugation (1100 rpm for 1 minute), removal of supernatant and, resuspension of the resin in BBS containing 10 mM imidazole. After the supernatant of the final wash was poured off, 0.5 ml of 1 M imidazole was added to each tube, vortex briefly, and transferred to a sterile microcentrifuge tube. The samples were then centrifuged at 14 krpm for 1 minutes and the supernatant transferred to a new microcentrifuge tube. Antibodies contained

15 in the supernatant were then analyzed for binding to SAP using a BIACORE (BIACORE, Uppsala, Sweden).

Selection and cloning of a recombinant polyclonal antibody complementary to IIT005.1.13 and IIT005.1.C.11 monoclonal antibodies

A monoclonal antibody designated IIT004.1.12 was selected from the

20 polyclonal library designated IIT004.1, biotinylated, and 15 µl of a 10^{-6} M solution was mixed with soluble recombinant SAP antigen (7.5 µl of a 10^{-7} M solution). This mixture was incubated for 15 minutes at room temperature. Fifteen microliters of each mixture was added to 50 µl of phage library IIT005.1 diluted in 1 ml panning buffer (40 mM Tris, 150 mM NaCl, 20 mg/ml BSA, 0.1% Tween 20, pH 7.5) and incubated overnight at 2-8°C. Final

25 concentrations were 10^{-8} M biotinylated monoclonal antibody and 5×10^{-10} M SAP. The sample was panned with avidin magnetic latex and plated as described in Example 4. The eluted phage were subjected to another round of selection using these conditions and the resulting polyclonal library was designated IIT005.1.C. Two monoclonal antibodies designated IIT005.1.13 and IIT005.1.C.11 were selected from the IIT005.1 and IIT005.1.C

30 libraries respectively, biotinylated and complexed with SAP using the conditions described

above. Complementary polyclonal antibodies were selected as described above from phage library IIT005.1 using monoclonal antibodies IIT005.1.13 and IIT005.1.C.11. These complementary polyclonal antibodies were designated IIT005.1.13.1 and IIT005.1.C.11.1 and were subcloned as described in Example 18 of US Patent Application Ser. No.

5   08/835,159.

Example 6

Specificity of Monoclonal/Polyclonal antibodies to SAP determined by Western blot and indirect immunofluorescence analysis

The specificity of monoclonal and polyclonal antibodies against *B. anthracis*,
10   Sterne strain SAP was visualized by Western blot analysis. Recombinant antibodies against SAP were tested for reactivity to recombinant SAP as well as to SAP isolated from the cleared culture supernatant of *Bacillus anthracis* Sterne strain. Cross reactivity to other *Bacillus* strains was also tested. Culture supernatant proteins and whole cell lysates of *B. anthracis*, Sterne strain, *B. cereus* OH599, *B. globigii* and *B. thuringiensis* 10792 equivalent
15   to 10^8 organisms were separated by electrophoresis in 4-20% TRIS-glycine SDS-polyacrylamide gels (Novex, San Diego, CA) under reducing conditions. The proteins were transferred to ProBlott™ membranes (Applied Biosystems, Foster City, CA) using 10 mM CAPS /10% methanol transfer buffer. The membranes were blocked in 10 mM TRIS, 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1% polyvinyl alcohol, 1% bovine serum
20   albumin, 0.1% sodium azide, pH 8.0 (Block buffer) for 1 h at room temperature.

The membranes were then incubated in 5 μg/ml of monoclonal or recombinant polyclonal antibody diluted in Block buffer for 1 h and then washed three times with 40 mM TRIS, 150 mM NaCl, 0.05% Tween 20, pH 7.5 (TBST) (Fisher Chemical, Pittsburgh, PA) for 5 min each. After washing, the membranes were incubated in rabbit
25   anti-mouse IgG (H&L)-alkaline phosphatase conjugate (Southern Biotechnology, Inc, Birmingham, AL) diluted 1:1000 in Block buffer. The membranes were washed three times with TBST for 5 min each and developed in a solution containing 0.2 M 2-amino-2-methyl-1-propanol (JBL Scientific, San Luis Obispo, CA), 0.5 M TRIS, 0.33 mg/ml nitro blue tetrazolium ((NBT) Fisher Scientific, Pittsburgh, PA) and 0.166 mg/ml 5-bromo-4-chloro-3-
30   indolyl-phosphate, p-toluidine salt.

The anti-SAP recombinant polyclonal antibodies reacted with recombinant SAP, SAP protein isolated from the culture supernatant, and the cell pellet of *B. anthracis*, Sterne strain. The antibodies did not react with any proteins in the culture supernatant or cell pellet of the other *Bacillus* species tested (*B. cereus* and *thuringiensis*). A goat anti-anthrax polyclonal serum was used to demonstrate cross-reactivity of *B. anthracis* antibodies with proteins of other *Bacillus* species (data not shown). Conjugates alone served as negative controls.

The specificity of antibodies against *B. anthracis*, Sterne strain was also tested by indirect immunofluorescence. Localization of SAP to the outer membrane of unencapsulated *B. anthracis*, Sterne strain was demonstrated using an indirect immunofluorescence technique. *B. anthracis*, *B. cereus*, and *B. thuringiensis* were washed and resuspended in PBS to yield 1×10^8 organisms per ml. Four microliters of the suspensions were applied to wells of an eight well microscope slide and allowed to air dry. The slides were lightly heated to fix the smears to the slide and covered with 0.1 mg/ml of antibody diluted in PBS containing 1% BSA. The smears were incubated with antibody for 1 h at 37°C in a moist chamber. After washing the slides three times by soaking in PBS for 5 min each, the smears were covered with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (H&L) F(ab')$_2$ (Zymed Laboratories, Inc., South San Francisco, CA) diluted 1:80 in PBS, 1% BSA, 0.05% Evans Blue (Sigma). The slides were incubated for 1 h at 37C in a moist chamber then washed as described above. After a final wash in deionized water, the slides were allowed to air dry in the dark. Coverslips were mounted using a 90% glycerol mounting medium containing 10 mg/ml *p*-phenylenediamine, pH 8.0.

The slides were examined for fluorescent organisms using an epifluorescence microscope with a 63x objective lens (Leitz Wetzler Germany). The recombinant polyclonal antibody (ITT005.1) demonstrated 4+ fluorescence with unencapsulated *B. anthracis* and did not react *with B. cereus*, or *B. thuringiensis*. Negative controls included fluorescein-conjugated antibody alone, and a murine polyclonal antiserum specific for *B. anthracis*, Sterne strain spore coat proteins.

Example 7

Sensitivity and Specificity of an ELISA plate assay for detection of *B. anthracis* SAP

This Example demonstrates that an ELISA assay using the reagents and methods of the invention are not only highly sensitive for *B. anthracis*, but are also highly
5   specific for this particular *Bacillus* species.

The sensitivity and specificity of various monoclonal/recombinant polyclonal antibody pairs were determined by performing a sandwich assay using biotinylated monoclonal antibodies and alkaline phosphatase-conjugated recombinant polyclonal antibodies. Assays were performed with NeutraAvidin or streptavidin coated plates, such as
10   Reacti-Bind™ streptavidin coated polystyrene 96 well plates (Pierce Chemical, Rockford, IL). After washing the 96 well plate with BBS (20 mM borate, 150 mM NaCl, 0.01% NaN₃, pH 8.0) containing 0.02% TWEEN-20, biotinylated monoclonal antibodies (50μL of 2.5μg/mL diluted in Block buffer (10 mM Tris, 150 mM NaCl, 10 mM MgCl₂, 0.1 mM ZnCl₂, 0.1% polyvinyl alcohol, 1% bovine serum albumin, 0.1% sodium azide, pH 8.0))
15   were added to the wells. The plate was incubated at room temperature for 1 hr.

The plate was then washed, after which various dilutions (10 ng/ml to 0.625 ng/ml) of soluble SAP antigen (50μL of recombinant SAP or SAP in culture supernatants (as prepared in Example 4) were added in duplicate to the biotinylated monoclonal wells. The plates were incubated for one hour at room temperature or overnight at 2-8°C, after which
20   the plate was washed. The appropriate recombinant polyclonal antibody-alkaline phosphatase conjugate (50μL of 2.5μg/mL diluted in Block) was added and incubated at room temperature for 1hr. After 1 hr, the plate was washed and developed using the ELISA Amplification System (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions.

25   Results from several assays are compiled in accompanying tables 2-5. These data indicate that the assays can detect less than 0.625 ng of SAP protein. This amount of SAP corresponds to approximately 1.8×10^3 *Bacillus anthracis* organisms per ml. Significantly, little or no cross reactivity to other related *Bacillus* species was detected.

Table 2: IIT005.1.C.11-BIOTIN WITH IIT005.1.C11.1-AP

B.anthracis culture (cfu/ml)	A490	SAP (ng/mL)	A490	Undiluted *Bacillus* species	A490
28330	3.4	10	3.55	*cereus*	0.28
14165	2.8	5	3.45	*thuringiensis*	0.27
7083	2.14	2.5	2.94	*subtilis niger*	0.55
3541	1.56	1.25	2.01	*subtilis*	0.51
1770	1.17	0.625	1.51	BHI broth	0.48
0	0.92	0	0.92	media	

Table 3: IIT005.1.13-BIOTIN WITH IIT005.1.13.1-AP

B.anthracis culture (cfu/ml)	A490	Undiluted *Bacillus* species	A490
28330	3.13	*cereus*	0.28
14165	2.21	*thuringiensis*	0.27
7083	1.5	*subtilis niger*	0.48
3541	0.99	*subtilis*	0.5
1770	0.78	BHI broth	0.423
0	0.55	media	

Table 4: IIT005.1.C.11-BIOTIN WITH IIT005.1-AP

B.anthracis culture (cfu/ml)	A490	SAP (ng/mL)	A490	Undiluted *Bacillus* species	A490
28330	2.87	10	3.4	*cereus*	0.09
14165	1.698	5	2.56	*thuringiensis*	0.14
7083	1	2.5	1.49	*subtilis niger*	0.13
3541	0.55	1.25	0.82	*subtilis*	0.14
1770	0.35	0.625	0.49	BHI broth	0.148
0	0.14	0	0.19	media	

Table 5: IIT005.1.13-BIOTIN WITH IIT005.1-AP

B.anthracis culture (cfu/ml)	A490	Undiluted Bacillus species	A490
28330	1.77	cereus	0.085
14165	0.99	thuringiensis	0.121
7083	0.54	subtilis niger	0.125
3541	0.34	subtilis	0.124
1770	0.23	BHI broth	0.125
0	0.14	media	

These results demonstrate that four different monoclonal/recombinant polyclonal antibody preparations exhibit great sensitivity for *B. anthracis* while not cross reacting with other *Bacillus* species.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.